

TESIS DOCTORAL
UNIVERSIDAD PABLO DE OLAVIDE



COENZYME Q₁₀ DEFICIENCY IN FAMILIAL HYPERCHOLESTEROLEMIA

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SEVILLA, 2019

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1.1. Abbreviations

AAV: Adeno-Associated Virus

ABCA1: ATP-Binding Cassette transporter A1

ABCG4: ATP-binding cassette subfamily G member 4

ABCG5: ATPbinding cassette subfamily G member 5

ABCG8: ATPbinding cassette subfamily G member 8

ACAT: Acetyl-CoA Acetyltransferase

ALT: Alanine Amino Transferase

AMPK: AMP-activated Protein Kinase

ANGPTL3: Angiopoietin Related Protein 3

apoA1: Apolipoprotein A1

apoB: Apolipoprotein B1

APOBEC: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ApoER2: apoE receptor 2

ARH: Autosomal Recessive Hypercholesterolemia Protein; LDLRAP1

AST: Aspartate Amino Transferase

ATG12: Autophagy-Related Protein 12

ATG5: Autophagy-Related Protein 12

ATP: Adenosine Triphosphate

C: Carbon

CATHEP B: Cathepsin B

CC: Compound C

CE: Cholesteryl esters

CEL: Carboxyl Ester Lipase

CETP: Cholesteryl Ester Transfer Protein

CK: Creatinine Kinase

CoA: Coenzyme A

COPII: Coat Complex Protein 2

CoQ₁₀: Coenzyme Q₁₀

CVD: Cardiovascular Diseases

CYP27: Sterol 27- hydroxylase
CYP7A1: Cholesterol 7 α -hydroxylase
Dil LDL: Fluorescent LDL Conjugates
DMEM: Dulbecco's Modified Eagle Medium
ECM: Extracellular Matrix
EGF-A: Epidermal Growth Factor-like A
ELISA: Enzyme-Linked ImmunoSorbent Assay
eNOS: endothelial NO Synthase
ER: Endoplasmic Reticulum
FBS: Fetal Bovine Serum
FC: Free Cholesterol
FCCP: Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FDA: Food and Drug Administration
FH: Familial Hypercholesterolemia
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
H: Hydrogen
HDL: High Density Lipoprotein
HMG-CoA: 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A
HMGCR: 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase
HPLC: High Pressure Liquid Chromatography
ICAM1: Intracellular Adhesion Molecule 1
IDL: Intermediate Density Lipoprotein
IDOL: Inducible Degradator of the LDL receptor
INSIG: Insulin Induced Gene
LCAT: Lecithin-Cholesterol Acyltransferase
LDL: Low Density Lipoprotein
LDLC: Low Density Lipoprotein Cholesterol
LDL-R: Low Density Lipoprotein Receptor
LIPA: Lysosomal Acid Lipase
LRP6: Low-density lipoprotein Receptor-related Protein 6
LSS: Lanosterol Synthase

LXR: Liver X Receptor

MAP LC3: Microtubule-associated proteins 1A/1B light chain 3B

miRNA: Micro Ribonucleotide Acid

MRC: Mitochondrial Respiration Chain

mRNA: Messenger Ribonucleic Acid

MTTP: Microsomal Triglyceride Transfer Protein

NADH: Nicotinamide adenine dinucleotide reduced form

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NAO: 10-N-nonyl Acridine Orange

NARC1: Neural Apoptosis Regulated Convertase 1

NF-kB: Necrosis Factor kappa Beta

NLRP3: NACHT, LRR and PYD domains-containing protein 3

NO: Nitric Oxid

NPC1: Niemann–Pick disease type C1

NPC1L1: Niemann–Pick C1-like 1

NPC2: Niemann–Pick disease type C2

O₂⁻: Superoxide anion

oxLDL: Oxydized LDL

PBS: Phosphate Buffered Saline

PCSK9: Proprotein Convertase Subtilisin/Kexin type 9

PDI: Protein Disulfide Isomerase

PPAR: Peroxisome Proliferator-Activated Receptor

qPCR: quantitative Polymerase Chain Reaction

RCT: Reverse Cholesterol Transport

RNA: Ribonucleic acid

RXR: Retinoid X Receptor

SCAP: Sterol Regulatory Element-Binding Protein cleavage-activating protein

SDS: Sodium dodecyl sulfate

SIM: Statin-Induced Myotoxicity

SIP: Site protease

SM: Squalene monooxygenase

SNP: Single Nucleotide Polymorphism

SRB1: Scavenger Receptor class B type 1

SREBPs: Sterol regulatory element-binding proteins

STAP1: Signal Transducing Adaptor Protein 1

TAG: Triacylglycerols

TLC: Thin Layer Chromatography

TMRM: Tetramethylrhodamine Methyl Ester

VCAM1: Vascular Adhesion Molecule 1

VDAC1: Voltage-dependent anion channel 1

VLDL: Very Low Density Lipoprotein

VLDLR: Very Low Density Lipoprotein Receptor

VSMC: Vascular Smooth Muscle Cells

$\Delta\Psi_m$: Mitochondrial Membrane Potential

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2. Resumen

La hipercolesterolemia familiar (FH) es un desorden genético autosómico dominante caracterizado por unos niveles elevados de lipoproteínas de baja densidad (LDL) en sangre y un aumento en el riesgo de enfermedades cardiovasculares tempranas como por ejemplo la aterosclerosis. En esta tesis, se ha estudiado la fisiopatología de la FH a nivel celular usando como modelo fibroblastos de la piel de pacientes afectados por FH los cuáles portan una mutación en el receptor de LDL, la cual es una de las mutaciones más comunes en la enfermedad.

Los fibroblastos de los pacientes afectados por la FH mostraron una menor captación de LDL asociado con una mayor cantidad de colesterol intracelular y una deficiencia de coenzima Q10 (CoQ10), advirtiendo una desregulación en la ruta del mevalonato.

La deficiencia secundaria de CoQ10 provoca un defecto en el funcionamiento mitocondrial así como la activación de procesos mitofágicos de forma continuada. La mitofagia constante altera el flujo autofágico e induce la activación del inflammasoma junto a un aumento de la producción de citoquinas en las células mutantes. Todas las alteraciones patológicas en los fibroblastos de FH fueron además reproducidos en un modelo de células endoteliales, las más afectadas en los procesos ateroscleróticos, mediante el silenciamiento del receptor de LDL.

Tanto el aumento intracelular de colesterol como la disfunción mitocondrial en fibroblastos con FH fueron parcialmente restaurados mediante la suplementación externa de CoQ10. La desregulación de la ruta del mevalonato en FH, incluyendo el aumento de expresión de enzimas colesterogénicas y la disminución de la expresión de genes relacionados con la biosíntesis de CoQ10, fueron también corregidos por el tratamiento de CoQ10.

Por ello, en estas tesis se establece que los bajos niveles de CoQ10 y la disfunción mitocondrial pueden jugar un papel crucial en la fisiopatología de la aterosclerosis temprana en FH y que el diagnóstico de la deficiencia de CoQ10 y las alteraciones mitocondriales también podrían ser importantes para establecer un tratamiento temprano suplementado con CoQ10 en pacientes afectados por FH.

3. Introduction

3.1. Cholesterol

Although the word “cholesterol” carries negative connotations for a lot of people, this substance actually has important physiologic functions. The reason is because cholesterol metabolism dysregulation can produce serious metabolic diseases and even death.

The word “cholesterol” comes from the Greek “khole” (bile) and “stereos” (stiff). Cholesterol was first identified in 1769 by François Poulletier de la Salle in human gallstones. However, it was not until 1815 that chemist Michel Eugène Chevreul named the compound "cholesterine" (Olson, 1998).

Since the past century, cholesterol has been related with numerous diseases such as atherosclerosis (Yin et al., 2015), Alzheimer’s disease (Banerjee & Mukherjee, 2018), cancer (Murai, 2015) or multiple sclerosis (Zhornitsky, McKay, Metz, Teunissen, & Rangachari, 2016). Despite its implications in those medical alterations, cholesterol is necessary and plays an essential role in the organism. Cholesterol most important functions are:

1. Regulator of the membrane structure and function (Xiaoyan Zhang et al., 2011).
2. Steroid hormones precursor (Midzak & Papadopoulos, 2014).
3. Bile acid and salt formation (Jing Wang, Bie, & Ghosh, 2016).
4. Vitamin D synthesis (Prabhu, Luu, Sharpe, & Brown, 2016).
5. Endocytosis (Yue & Xu, 2015).
6. Cell signaling (Fukui, Ferris, & Kahn, 2015).
7. Electrical impulse enhancer (Saher & Stumpf, 2015).

Cholesterol is a 27-carbon polycyclic lipid molecule organized in four fused rings in a planar conformation. It has one unsaturated double bond (C5-C6), one β -hydroxyl substitution (C3) and a simple 8-carbon aliphatic tail (**Figure 1**). Although its lipid molecule nature, cholesterol cannot be used as energy source since no enzymes exist that can break the sterol core down to its original acetyl-Coenzyme A (CoA) units.

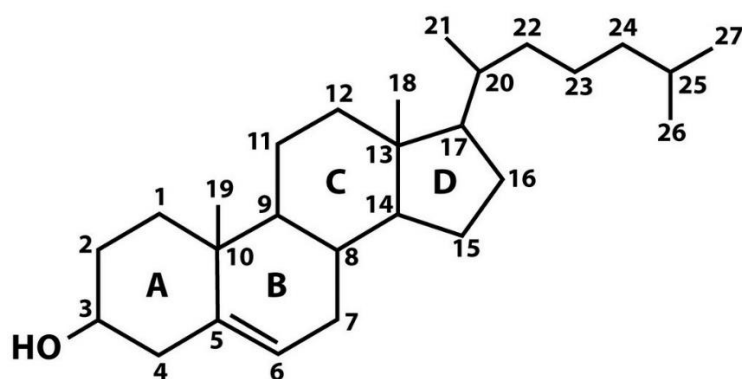


Figure 1. Cholesterol structure. Cholesterol is a 27-carbon, planar, 3β -hydroxylated sterol molecule. These structural features are essential, since minor modifications block cholesterol biological functioning.

Cholesterol is an hydrophobic molecule and therefore it is exclusively found in membranes and other lipid and/or lipid-protein complexes (Hofmann et al., 2014). Within membranes, cholesterol interacts with phospholipids and sphingolipid fatty acyl chains while simultaneously increasing membrane bilayer rigidity and reducing water and ion membrane permeability (Choubey, Kalia, Malmstadt, Nakano, & Vashishta, 2013). At the subcellular level, cholesterol is heterogeneously distributed, with only up to 1% of total cell cholesterol present in the endoplasmic reticulum (ER) (Stevenson, Huang, & Olzmann, 2016), almost 80% in the plasma membrane and the rest is organized in lipid droplets or lipoproteins (Das, Brown, Anderson, Goldstein, & Radhakrishnan, 2014). In the plasma membrane, cholesterol can reach the 20% of total lipid mass and appears to be critical for its organization and function (Rituper et al., 2013). In fact, membrane microdomains called “lipid rafts”, which are dynamic protein/lipid assemblies that drift in the liquid-disordered membrane bilayer and are important for extracellular ligand binding as well as for intracellular signal transduction (Sonnino & Prinetti, 2013), can only be formed when membrane cholesterol concentration achieves a 10% threshold content. Taken together, these reasons make cholesterol an essential molecule while its negative effects reside in its overaccumulation or rarely in its absence.

3.2. Cholesterol homeostasis

3.2.1. The mevalonate pathway

3.2.1.1. Cholesterol biosynthesis

Cholesterol is either supplied from the diet (exogenous) or synthesized *de novo* by many cells of the body (endogenous). The dietary intake of cholesterol is limited, and therefore the physiological requirements for cholesterol are provided mostly through *de novo* synthesis. Almost all cells are involved in the synthesis of cholesterol, with the liver accounting for as much cholesterol as non-hepatic tissues combined (Dietschy, Turley, & Spady, 1993).

The mevalonate pathway is one of the most important metabolic networks in the cell; it provides essential cell constituents, including cholesterol, some of its branches produce key metabolites, such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, both involved in the biosynthesis of terpenes and terpenoids necessary for normal cell metabolism (Marschalek et al., 2015). In the last steps of the cholesterol pathway, the vitamin D precursor, 7-dehydrocholesterol (7-DHC), is also synthesized (Kühn, Hirche, Geissler, & Stangl, 2016). Importantly, isoprene intermediates in the mevalonate pathway generate a variety of other bioactive molecules like dolichol and ubiquinone.

Cholesterol is synthesized from its precursor unit acetyl-coenzyme A (CoA) via a complex metabolic pathway (Joseph L. Goldstein & Brown, 1990). Eighteen acetyl CoA units containing 36 carbon (C) atoms are utilized to synthesize one molecule of cholesterol; which contains 27 C atoms and 46 hydrogen (H) atoms. Seven H atoms are incorporated into the cholesterol molecule directly from water while another 15 atoms are inserted from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. H atoms from water may also become incorporated into substrates that later generate cytosolic acetyl CoA used for cholesterol biosynthesis. The 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase (HMGCR) is the rate-limiting enzyme in cholesterol synthesis (Rodwell, Nordstrom, & Mitschelen, 1976).

Cholesterol is synthesized in the ER by the action of over 30 enzymes organized in the mevalonate pathway (**Figure 4**) whose first part involves four fundamental steps:

1. Condensation acetyl-CoA units to form HMG-CoA.
2. HMG-CoA reduction mediated by NADPH -oxidase to generate mevalonate.

3. Conversion of mevalonate into activated isoprenoids 3-isopentenyl pyrophosphate and dimethylallyl pyrophosphate.

4. Polymerization of six isoprenoids units into squalene.

Next, linear squalene undergoes series of oxygenation and cyclization to form lanosterol. From this precursor, cholesterol is synthesized in a 19-step process involving the activity of nine different enzymes. The last steps of cholesterol synthesis have been divided into two pathways and can proceed via lanosterol and 7-dehydrocholesterol (Kandutsch–Russell pathway) (KANDUTSCH & RUSSELL, 1960) or via desmosterol to cholesterol (Bloch pathway) (Bloch, 1992). These pathways share the same enzymatic steps but differ in the stage at which the C-24 bond is reduced.

3.2.1.2. Coenzyme Q₁₀ biosynthesis

One of the most important molecules in the cell that requires a mevalonate pathway metabolite is the ubiquinone. Ubiquinone or coenzyme Q₁₀ (CoQ₁₀) is known as a versatile molecule because of its many functions on the organism. CoQ₁₀ is a benzoquinone ring conjugated to a ten units long isoprenoid chain making it a hydrophobic molecule that can act as a great electron carrier and free radical scavenging antioxidant (Acosta et al., 2016). CoQ₁₀ is mainly required in the mitochondria as electron carrier in the mitochondrial respiratory chain (MRC). MRC provides, through oxidative phosphorylation, the capacity to synthesize adenosine triphosphate (ATP) which is essential for cellular function. Only just a 30% (Gnaiger, 2009) of CoQ₁₀ is protein bound which are thought to be part of the MRC. The free mitochondrial CoQ₁₀ pool may serve to other functions like lipophilic antioxidant (Bentinger, Brismar, & Dallner, 2007), permeability transition pore opening regulator and maintenance of body temperature via its role as a cofactor for the mitochondrial uncoupling proteins (López-Martín et al., 2007).

The biochemical pathway responsible for CoQ₁₀ biosynthesis is still incompletely identified. In mammals, the precursor of the quinone ring is only the 4-hydroxybenzoate, which is derived from tyrosine through an uncharacterized set of reactions. On the other hand, the isoprenoid tail biosynthesis is well known and it is synthesized through the mevalonate pathway, which is common also to cholesterol biosynthesis (Bentinger, Tekle, & Dallner, 2010). Specifically, after the decarboxylation of mevalonate pyrophosphate yields isopentenyl pyrophosphate, which is the precursor of farnesyl pyrophosphate and also the building block

for the biosynthesis of dolichol and the side chain of CoQ₁₀. Therefore, alterations in the mevalonate pathway not only will affect cholesterol but CoQ₁₀ related cellular mechanisms.

The CoQ₁₀ biosynthesis involves a multi-subunit protein complex (Coq1 to Coq10) localized in the inner mitochondrial membrane (Tran & Clarke, 2007). The polyisoprenoid chain is synthesized by Coq1, and its length, depending on the species, may range from 6 (CoQ₆) to 10 (CoQ₁₀) isoprene units. This isoprene product is then condensed to a 4-hydroxybenzoate-derived benzoquinone ring by Coq2 (Forsgren et al., 2004); while Coq3, Coq5, Coq6, and Coq7 are involved in methylation, decarboxylation, hydroxylation and deamination reactions (Tran & Clarke, 2007). Coq8 is an atypical protein kinase essential for phosphorylation of Coq3, Coq5 and Coq7 (Pierrel et al., 2010). Coq9 is a lipid-binding protein necessary to stabilize Coq7 and apparently controls the deamination of CoQ₁₀ intermediates that derive from para-aminobenzoic acid (He et al., 2015). Coq10 is a polypeptide that binds CoQ₁₀ and facilitates both *de novo* CoQ₁₀ biosynthesis and respiratory electron transport probably directing the localization of CoQ₁₀ within the mitochondrial membrane (Barros et al., 2005). Finally, the function of Coq4 is still unknown, but there is evidence that it is required for the assembly and stability of the CoQ₁₀ biosynthetic complex (Barros et al., 2005).

Statins, the famous cholesterol-reducing drug, are effective because they are capable to inhibit the mevalonate pathway through HMG-CoA inhibition, therefore inhibiting all the cholesterol and related metabolites biosynthesis. Although it is a great choice in most hypercholesterolemia pathologies, statins have been related with myalgia. Several hypothesis support the idea of reduced production of farnesyl pyrophosphate, an intermediary used in the biosynthesis of CoQ₁₀. Statins will be discussed in the following sections of the thesis.

3.2.2. Cholesterol intestinal absorption

In addition to being endogenously synthesized, cholesterol is incorporated into the cell from plasma lipoproteins by cell surface receptor mediated endocytosis. Cholesterol is a hydrophobic molecule, and its intestinal absorption requires emulsification, hydrolysis (of dietary esterified cholesterol), micellar solubilization, and uptake within enterocytes. Furthermore, intracellular cholesterol is partly re-esterified and finally delivered to the bloodstream. Cholesterol present in the intestinal lumen derives from several sources, including diet, bile, intestinal secretion and desquamated epithelial cells. In humans consuming Western type diets, 300–500 mg dietary cholesterol enters the intestinal lumen per day,

whereas the contribution of biliary cholesterol has been estimated to be approximately 800–1200 mg per day.

In healthy humans normally approximately 50% of intestinal cholesterol is absorbed (Sudhop et al., 2002) and represents the 50% of the cholesterol in the organism. Cholesterol entering the small intestinal lumen, and subsequently absorbed by the enterocytes (**Figure 2**), is derived from three major sources: diet, bile, and intestinal epithelial sloughing (D. Q.-H. Wang, 2007). The duodenum and proximal jejunum are the major sites of absorption (Arnesjö, Nilsson, Barrowman, & Borgström, 1969). For the cholesterol to be absorbed, micellar solubilization must take place (Woollett et al., 2006). Dietary cholesterol is partly esterified (<15%) in comparison to biliary cholesterol which is predominantly unesterified. This requires the dietary cholesteryl esters (CE) to be de-esterified by pancreatic carboxyl ester lipase (CEL) before cholesterol can be packed and transported to the brush border of enterocytes (van der Wulp, Verkade, & Groen, 2013). It must be noted that since the biliary pool of unesterified cholesterol is larger than the esterified dietary pool of cholesterol, targeted inhibition of the pancreatic lipolytic enzymes becomes ineffective in reducing cholesterol absorption (D. Q.-H. Wang, 2007). Two enzymes mainly control the intestinal uptake and transport process of sterols: acetyl-CoA acetyltransferase (ACAT), which facilitates intracellular cholesterol esterification, and the microsomal triglyceride transfer protein (MTTP), which is involved in intestinal chylomicron assembly (D. Q.-H. Wang, 2007).

The Niemann–Pick C1-like 1 (NPC1L1) transporter facilitates the uptake of cholesterol and plant sterols/ stanols (Davis & Altmann, 2009). NPC1L1 is located in the brush border membrane of enterocytes in the proximal jejunum of the small intestine. The suppression of the NPC1L1 gene reduces a 70 and 90 % in cholesterol and plant sterol/stanol absorption, respectively (Davis et al., 2004). The non-effluxed intracellular cholesterol translocates to the endoplasmatic reticulum and subsequently gets esterified by ACAT (R. G. Lee, Willingham, Davis, Skinner, & Rudel, 2000). The esterified cholesterol is then incorporated into chylomicron together with triacylglycerols (TAG), phospholipids, and to apolipoprotein B48 by MTTP to be delivered to the lymph (Gordon & Jamil, 2000). In addition, enterocytic cholesterol can be transferred to apolipoprotein A1 (apoA1) high density lipoprotein (HDL) particles via ATP-binding cassette transporter A1 (ABCA1) (Brunham et al., 2006).

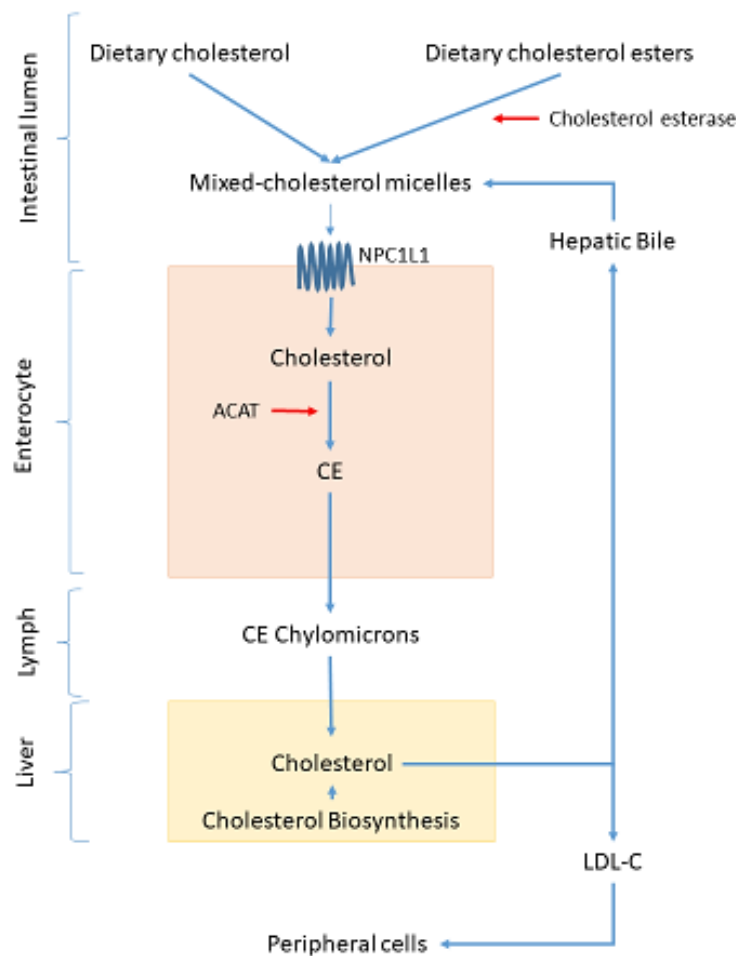


Figure 2. Cholesterol absorption and biosynthesis pathways. Dietary cholesterol entering the proximal intestinal lumen is composed of both cholesterol and cholesterol esters. Cholesterol esterase catalyses the hydrolysis of CE to free cholesterol and contributes to the formation of lysolecithin-containing micelles. Cholesterol in micelles both from the dietary source and from hepatic cholesterol are subsequently available for uptake via the proximal intestinal lumen. The process of cholesterol uptake is mediated by a protein transport mechanism, as evidenced by the kinetic processes involved, the sterol specificity and the fact that the drug ezetimibe inhibits cholesterol uptake. The NPC1L1 is the crucial mediator in the cholesterol absorption and has been shown to reside on the apical surface of the intestinal enterocytes. Cholesterol re-esterification occurs within the enterocytes via ACAT and the cholesterol is packaged into chylomicrons and secreted into the lymph. Owing to the high amounts of NPC1L1 expression in the liver, it is likely that the cholesterol transporter contributes to the re-uptake of cholesterol from micelles in bile and this process is likely to be inhibited by ezetimibe. Simultaneously, the liver hepatocytes synthesize cholesterol via a complex process involving at least 30 enzymes.

NPC1L1; Niemann-Pick C1-like protein 1, ACAT; Acyl-CoA Cholesterol Acyltransferase, FC; Free cholesterol, HDL; High Density Lipoprotein, LDL-C; Low Density Lipoprotein Cholesterol; CE, Cholesterol esters

3.2.3. Cholesterol cell uptake

As the chylomicrons reach the circulation via the lymph some of the triglycerides are hydrolyzed by lipoprotein lipase and the chylomicron remnants are taken up by hepatocytes. In turn, hepatocytes secrete lipids in very low density lipoproteins (VLDL) particles that are processed in the circulation into low density lipoproteins (LDL), the main lipoprotein that delivers cholesterol to the peripheral cells.

Lipoprotein transport of cholesterol in plasma plays a physiological role for essential energy production, cell membrane, and hormone synthesis. Cells readily utilize cholesterol by internalizing lipoprotein ligands containing chylomicron, LDL, intermediate-density lipoprotein (IDL), or VLDL mediated by the LDL receptor (LDL-R) family of membrane receptors. Brown and Goldstein first dissected the now prototypical LDL-R pathway (**Figure 3**) (J L Goldstein & Brown, 1974a), demonstrating that specific cell surface receptors are required for extracellular ligand endocytosis (Brown & Goldstein, 1986). In this model, circulating LDL binds to LDL-R on the cell surface and are incorporated, as a whole lipoprotein-receptor complex, via clathrin-coated vesicles, into endolysosomal compartments for further processing. At this level, LDL-R is recycled back to the surface, whereas LDL particles are fully degraded into their individual components. More specifically, LDL-derived cholesteryl esters are de-esterified to form free cholesterol and fatty acids by the action of lysosomal acid lipase. LDL-R-mediated endocytosis has provided much of our understanding of lipoprotein clearance, and its defect is a major cause of familial hyperlipidemia and a fundamental risk factor for cardiovascular disease (Go & Mani, 2012).

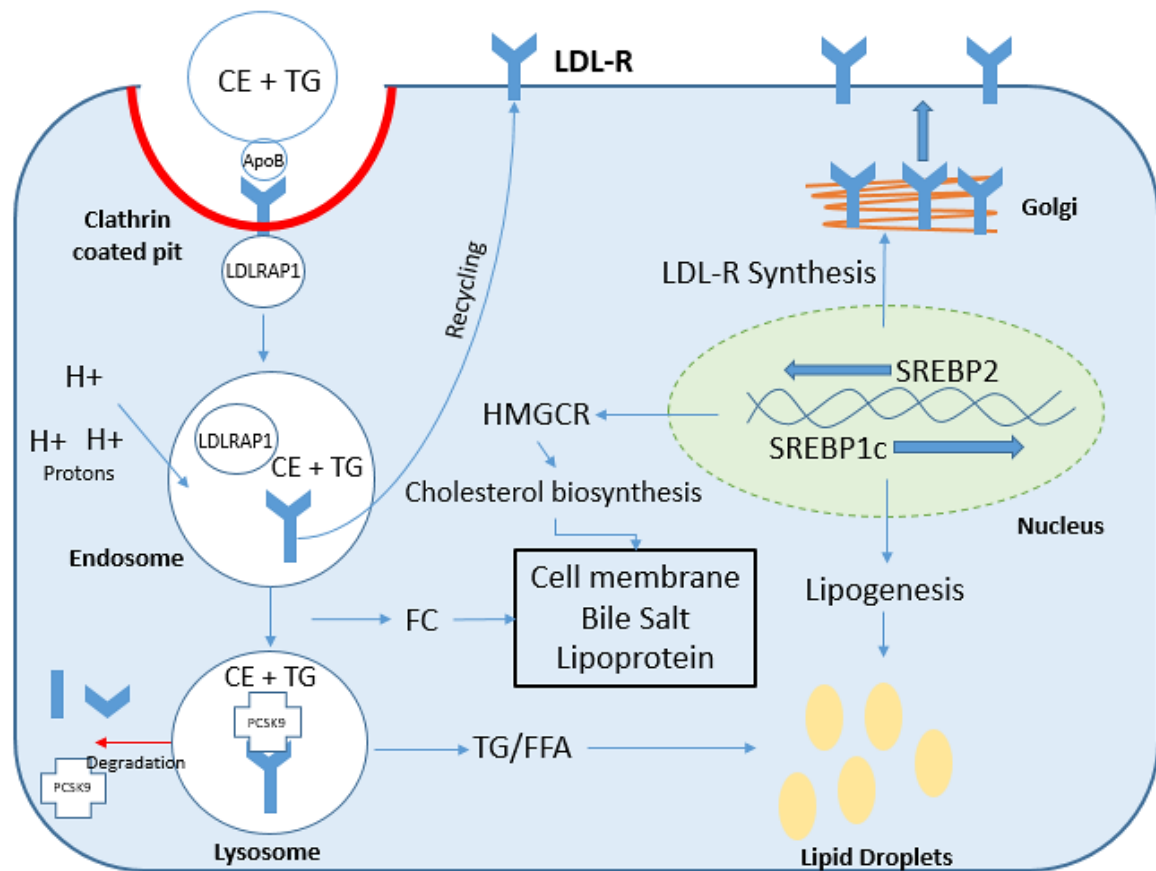


Figure 3. Cellular cholesterol homeostasis. Vesicular uptake of lipoproteins is essential for lipoprotein and lipid metabolism. This process is regulated by the LDL-R family of proteins. Recognition of apolipoproteins by the receptor at neutral pH initiates the internalization, followed by LDLRAP1 (LDL-R adaptor protein; also known as ARH) binding of the cytoplasmic NPxY motif and clustering of receptor-ligand complexes into clathrin-coated pits. Coated vehicle dispenses to endosomes, in which acidic condition activates the release of internalized ligands from the receptor. Released ligand particles travel further to lysosome, in which ligand is degraded by enzyme. The receptors recycle back to the cell surface. Internalized cholesterol reduces cholesterol biosynthesis and LDL-R transcription by inhibiting SREBP-2. PCSK9 binds to LDL-R, which is targeting LDL-R to lysosome for degradation. *De novo* lipogenesis is also reduced by inhibition of SREBP-1c. TG undergoes adipogenesis to form lipid droplet.

LDL-R, Low Density Lipoprotein Receptor; LDLRAP1, LDL-R Adaptor protein 1; SREBP, Sterol regulatory element-binding protein; PCSK9, Proprotein convertase subtilisin/kexin type 9; TG, Triglycerides; CE, Cholesterol Esters; FC, Free Cholesterol; FFA, Free Fatty Acids; HMGCR, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase

LDL-R is a cell membrane glycoprotein that functions in the binding and internalizing of circulating cholesterol-containing lipoprotein particles. LDL-R is ubiquitously expressed and is a key receptor for maintaining cholesterol homeostasis in mammals. LDL-R-mediated endocytosis is essential for lipoprotein and lipid metabolism (J L Goldstein & Brown, 1974a). Recognition of apolipoprotein B (apoB) of LDL particles occurs with a stoichiometry of a single copy of apoB per one LDL particle per receptor monomer (Wiklund, Dyer, Tsao, & Curtiss, 1985). VLDL, IDL, HDL, and chylomicron remnant are also recognized by LDL-R at neutral pH (Innerarity, Mahley, Weisgraber, & Bersot, 1978). Coated vesicles fuse with endosomes with LDL-R and LDL-R adaptor protein (LDLRAP1), connecting the LDL-R family protein and the endocytic machinery; thereby, acidic condition activates dissociation of internalized ligands. Released ligand particles further travel to the lysosome, in which the ligand is degraded by enzyme, while the receptors recycle back to the cell surface. LDL particles trigger three steps after internalization (Brown & Goldstein, 1976) :

1. Reduces gene expression of HMGCR to suppress cholesterol biosynthesis.
2. Enhances activity of ACAT to reduce toxic free cholesterol.
3. Suppresses LDL-R synthesis to reduce LDL uptake via sterol regulatory element-binding proteins (SREBP).

The LDL-R dissociates from LDL in the early endosome and is recycled back to the plasma membrane by vesicular mechanisms that depend on small G-proteins from the Rab family, such as Rab8 (Linder et al., 2007) and Rab11 (Hölttä-Vuori, Tanhuanpää, Möbius, Somerharju, & Ikonen, 2002). The non-recycled contents of the early endosome, namely cholesterol, proceed to the late endosome/ lysosome and upon release, are delivered to other membranes, such as the plasma membrane, ER, and mitochondria. Two proteins, Niemann–Pick disease, type C1 and C2 (NPC1 and NPC2, respectively), are crucial for moving cholesterol out of the late endosomal system and into the cytosol. A deficiency of either protein leads to Niemann–Pick type C disease, which is characterized by an accumulation of free cholesterol in late endocytic organelles (Ory, 2004). Although the exact roles of these proteins are not clear, it is predicted that NPC2, a small soluble protein, accepts cholesterol in the late endosomal lumen and transports it to membrane-bound NPC1, which helps transfer cholesterol out of the endosomal system (M. L. Wang et al., 2010).

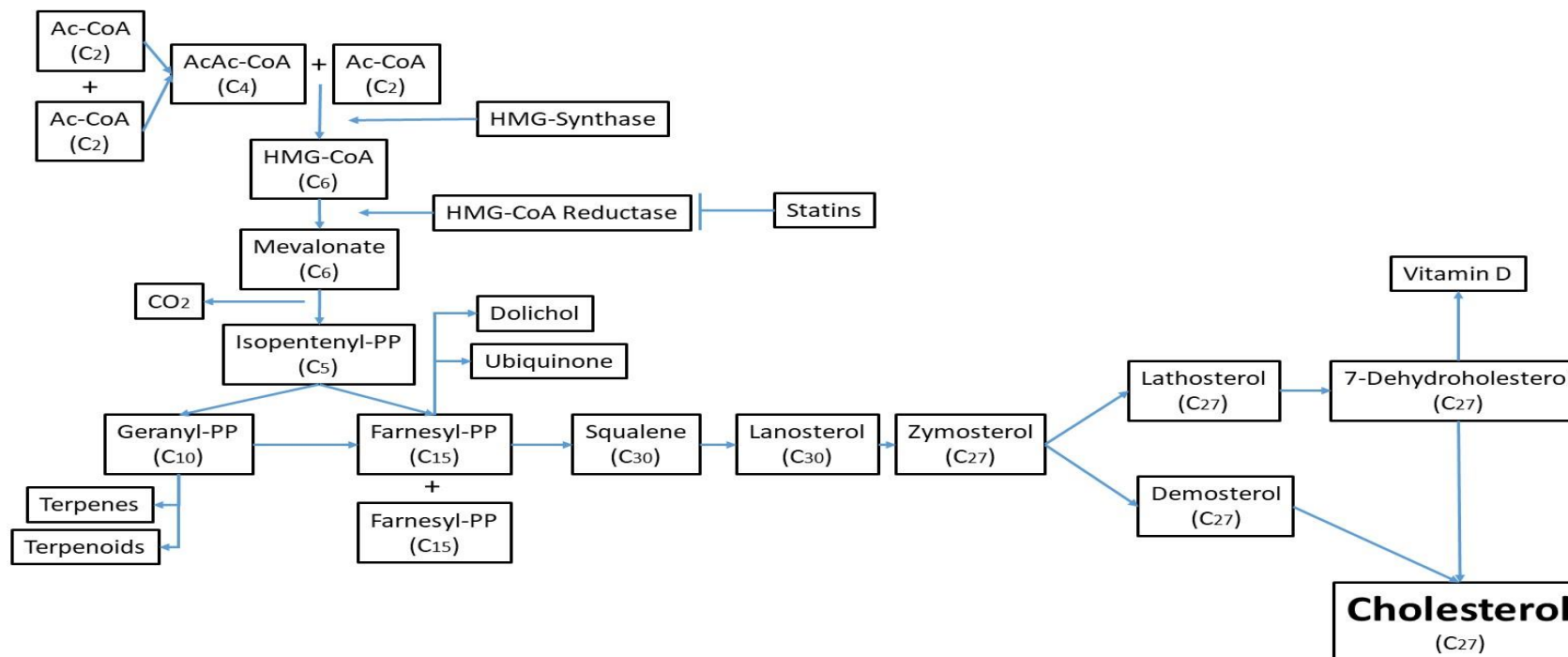


Figure 4. Cholesterol synthesis in the mevalonate pathway. First, HMG-CoA and mevalonate are formed from acetyl-CoA. The conversion of HMG-CoA into mevalonate is catalyzed by HMG-CoA reductase. Mevalonate is converted into activated isoprenoids isopentenyl pyrophosphate and methylallyl pyrophosphate (not shown), which, in turn, originate farnesyl pyrophosphate and squalene. The subsequent cyclization and oxygenation of squalene to lanosterol involves the action of squalene monooxygenase and lanosterol synthase and requires molecular oxygen and NADPH. Finally, the generation of cholesterol involves lanosterol: 1) demethylation at C4 α , C4 β , and C14; 2) isomerization of the Δ 8(9) double bond to a Δ 7 double bond; 3) desaturation to form a Δ 5 double bond; and 4) reduction of Δ 14, Δ 24, and Δ 7 double bonds. Importantly, the reduction of Δ 24 double bond, catalyzed by 3 β -hydroxysterol Δ 24-reductase (DHCR24) can happen at any level below lanosterol, originating two parallel pathways and resulting in either 7-dehydrocholesterol or desmosterol, which are reduced to cholesterol by DHCR7 or DHCR24, respectively (Cortes et al., 2014).

HMG-CoA, 3-hydroxy-3-methyl-glutaryl CoA; NADPH, Reduced Nicotinamide adenine dinucleotide; DHCR7, 7- dehydrocholesterol reductase; DHCR24, 24-dehydrocholesterol reductase; CoA, Coenzyme A; Ac-CoA; Acetyl Coenzyme A; PP, Pirophosphate

3.2.4. Cholesterol regulation

3.2.4.1. Cellular consequences of cholesterol accumulation

Cholesterol may be deleterious or even lethal for cells and many protective mechanisms have evolved to prevent this cellular toxicity. The underlying causes of cholesterol cytotoxicity are not completely clear. A physiological free cholesterol (FC)/phospholipid ratio in cellular membranes is necessary to maintain proper membrane fluidity, or more precisely, a proper range of membrane fluidities (K Simons & Ikonen, 2000). The degree of saturation of the fatty acyl moieties of membrane phospholipids is the major determinant of the fluidity of lateral membrane domains, which consist of well-packed, detergent-resistant liquid-ordered rafts and more fluid, detergent-soluble liquid-crystalline regions (Kai Simons & Ehehalt, 2002). Nonetheless, the interaction of the hydrophobic rings of cholesterol with these fatty acyl chains has important effects (Maxfield & Wüstner, 2002). In particular, the ability of cholesterol to pack tightly with saturated fatty acyl groups of membrane phospholipids is critical for the formation of liquid-ordered rafts (K Simons & Ikonen, 2000). When the FC/phospholipid ratio rises above a physiological level, the liquid-ordered rafts may become too rigid, and the liquid-crystalline domains may begin to lose their fluidity. These alterations affect certain integral membrane proteins that require conformational freedom for proper function and that can be inhibited by a high FC/phospholipid ratio (Yeagle, 1991). High FC levels might therefore be proposed to kill cells in part by inhibiting one or more integral membrane proteins whose function is blocked or altered under conditions of high membrane rigidity (Ira Tabas, 2002). Models for FC-induced cell death are summarized in **Table 1**.

Excess membrane cholesterol may also disrupt the function of signaling proteins that reside in membrane domains (Pierini et al., 2003). One interpretation of these data is that excess plasma membrane cholesterol disrupts the function of certain signaling molecules that normally reside in non-raft domains. Experiments in vitro with model membranes suggest that, in membranes already enriched in sphingolipids, increasing the FC concentration even modestly above the physiological concentration can actually suppress the formation of membrane domains (Milhiet, Giocondi, & Le Grimellec, 2002).

Table 1. Potential mechanisms of FC-induced cytotoxicity. Modified from (Ira Tabas, 2002)

Event	Consequence	Reference
Loss of membrane fluidity	Dysfunction of integral membrane proteins and exocytosis	(Bogan, Xu, & Hao, 2012)
Disruption of membrane domains	Disruption of signaling events	(Tomita et al., 2014)
Induction of apoptosis	Caspase-mediated cell death	(Zhu et al., 2014)
Intracellular cholesterol crystallization	Organelle disruption, inflammation	(Corr, Cunningham, & Dunne, 2016)
Formation of toxic oxysterols	Oxidative damage, carcinogenesis	(Jusakul, Yongvanit, Loilome, Namwat, & Kuver, 2011)
Alteration of gene expression	Change in balance of survival vs. death proteins	(LIM, PARAJULI, DUONG, CHOI, & HAN, 2014)

Other mechanisms of cellular toxicity associated with FC accumulation include intracellular cholesterol crystallization, oxysterol formation (Björkhem, 2002) and triggering of apoptotic signaling pathways (I Tabas, 1997). Cholesterol crystals form when the FC/phospholipid ratio reaches a very high level. Intracellular cholesterol crystals can probably damage cells by physically disrupting the integrity of intracellular structures (Corr et al., 2016). Excess intracellular FC accumulation can also promote the oxidation of cholesterol to oxysterols, some of which may be cytotoxic (Khatib & Vaya, 2014). Finally, FC overloading of macrophages can trigger a series of apoptotic pathways (Afonso et al., 2014) which leads to the major cause of cardiovascular disease: atherosclerosis.

3.2.4.2. Cholesterol biosynthesis regulation

The regulation of cholesterol homeostasis involves a complex interplay between a growing list of proteins that act to sense and respond to changing levels of cellular cholesterol (**Figure 5**). These proteins may be regulated by cholesterol itself or by certain cholesterol intermediates or derivatives.

While the majority of cellular cholesterol resides in the plasma membrane, most of the cholesterol homeostatic machinery is located in the ER. Among these are the sterol regulatory element-binding protein 2 (SREBP2), the master transcription factors in cholesterol homeostasis, and its associated regulatory proteins. These include SCAP (SREBP cleavage-activating protein), which complexes with SREBP2 in the ER membrane when cholesterol levels are low and escorts it to the Golgi for proteolytic activation; INSIG 1 and 2 (insulin-induced gene), which retains SCAP in the ER when cellular cholesterol levels are sufficient or in excess. Most cholesterol synthesis enzymes, including the two rate-limiting enzymes HMGCR and squalene monooxygenase (SM), also reside in the ER, as well as the cholesterol esterification enzyme ACAT, which esterifies excess cholesterol for storage (Howe et al., 2016).

The SREBP family of transcription factors control cholesterol and lipid metabolism and play critical roles during adipocyte differentiation and insulin-dependent gene (P. J. Espenshade, 2006; Horton, Goldstein, & Brown, 2002). The family consists of three different SREBP proteins, SREBP1a, SREBP1c and SREBP2. The SREBPs are synthesized as large precursor proteins that are inserted into the ER membrane through two membrane-spanning domains (Bengoechea-Alonso & Ericsson, 2007). The membrane-associated proteins are transcriptionally inactive. In the ER, the C terminus of the SREBP interacts with SCAP, which functions as a sterol sensor (Edwards, Tabor, Kast, & Venkateswaran, 2000; Joseph L. Goldstein, DeBose-Boyd, & Brown, 2006). In sterol-depleted cells, SCAP escorts the SREBPs from the ER to the Golgi, where they are processed by two membrane-associated proteases, the site 1 and site 2 proteases (SIP), which release the mature forms of the proteins. These transcriptionally active fragments of the SREBPs are translocated to the nucleus, where they bind to the promoters of SREBP target genes, including genes involved in the synthesis and metabolism of cholesterol. In addition, transcription of the genes encoding SREBP1c and SREBP2 is enhanced by SREBPs by a feed-forward mechanism that requires functional SREBP binding sites in the promoters of these genes. When cholesterol builds up in the ER membranes, the SCAP/SREBP complex is retained in the ER, the proteolytic activation of SREBPs is stopped, and the expression of SREBP target genes declines. Thus, the amount of

cholesterol in cells is controlled by a tightly regulated feedback system. This regulatory system extends beyond the SREBPs, since HMGCR, the rate-limiting enzyme in cholesterol synthesis, is also controlled by sterol-dependent proteolysis (Joseph L. Goldstein et al., 2006). The feedback regulation of SREBPs is complemented by the rapid ubiquitin-dependent degradation of the active transcription factors in the nucleus.

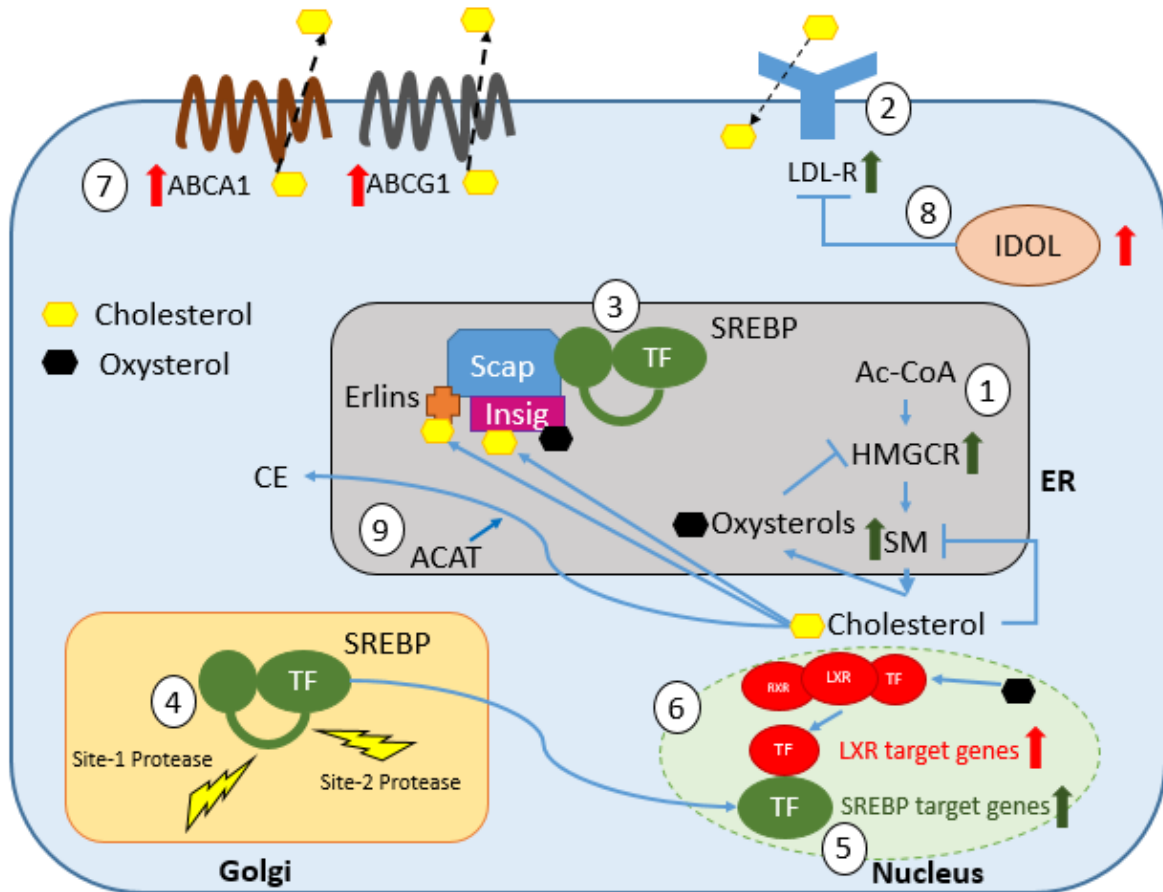


Figure 5. Interplay of cholesterol homeostatic machinery. Cholesterol is synthesized from Ac-CoA in the ER (1) or taken up through the LDL-R (2). When sterol levels are low, INSIG dissociates from SCAP, enabling SCAP to escort SREBP (3) to the Golgi for processing by Site-1 and Site-2 proteases (4), SCAP is recycled back to ER. This releases an SREBP TF that translocates to the nucleus and upregulates SREBP target genes (5). These include HMGCR, SM and LDL-R. When sterol levels are high, cholesterol negatively regulates SM and oxysterols negatively regulate HMGCR, causing their degradation. Cholesterol binds to SCAP and erlins -1 and -2, and oxysterols bind to INSIG, causing the retention of SCAP/SREBP in the ER. Oxysterols, also act as ligands for the LXR-RXR heterodimer, releasing the LXR TF and upregulating transcription of LXR target (6) involved in cholesterol efflux. These include ABCA1 and ABCG1, which synergise to export cholesterol from the cell (7), and IDOL, which mediates degradation of LDL-R (8). Excess cholesterol can also be esterified by ACAT for storage in an inactive form (9).

ER; Endoplasmic Reticulum, LDL-R; Low Density Lipoprotein Receptor, INSIG; Insulin induced gene 1, SCAP; SREBP cleavage-activating protein, SREBP; Sterol regulatory element-binding protein cleavage-activating protein, TF; Transcription factor, SM; Squalene Monooxygenase, LXR; Liver X Receptor, ACAT; Acyl-CoA cholesterol acyltransferase, RXR; Retinoid X Receptor, IDOL; Inducible Degradator of the LDL receptor; Ac-CoA, Acetyl Coenzyme A

The sterol-dependent regulation of SCAP and HMGCR is dependent on the sterol-sensing domains found in the transmembrane domains of these proteins. In the case of SCAP, direct binding of cholesterol to its sterol-sensing domain induces a conformational change in the protein (Feramisco et al., 2005). As a result, SCAP can no longer interact with the coat complex protein 2 (COPII) coat proteins Sar1 and Sec23/24, and the SCAP/SREBP complex is not incorporated into COPII-coated vesicles (Sun, Li, Goldstein, & Brown, 2005). Consequently, SREBPs are not transported to the Golgi or activated. The sterol-mediated inhibition of SCAP binding to COPII proteins is enhanced by both INSIG1 and INSIG2 (Dong, Tang, & Chen, 2012). INSIGs are resident ER proteins that interact with both SCAP and HMGCR in a sterol-dependent manner. Binding of cholesterol to SCAP promotes the interaction between SCAP and INSIG, thereby retaining the SCAP/SREBP complex in the ER and preventing activation of SREBPs. Thus, INSIGs are negative regulators of SREBP function and it appears that INSIGs enhance the response to cholesterol by promoting the binding of cholesterol to SCAP.

INSIG1 is the dominant isoform, however, both INSIG1 and INSIG2 block the translocation of the SCAP/SREBP complex in response to sterols (Joseph L. Goldstein et al., 2006). INSIG1 is an SREBP target gene and the induction of INSIG1 mRNA in response to insulin treatment is an indirect effect of the insulin-dependent induction of the SREBP1c gene, related to lipid synthesis (Guo, Bell, Mischel, & Chakravarti, 2014). Thus, SREBP induces the expression of its own negative regulator. INSIG2 is expressed at low levels, is not regulated by SREBPs and it is stable. By contrast, INSIG1 is rapidly degraded by the ubiquitin-proteasome system (UPS) in sterol-depleted cells (Gong, Lee, Lee, et al., 2006; Gong, Lee, Brown, Goldstein, & Ye, 2006; J. N. Lee, Gong, Zhang, & Ye, 2006). The ubiquitination and degradation of INSIG1 is negatively regulated by sterols, since INSIG-1 is stabilized when bound to the SCAP/SREBP complex in the presence of cholesterol (Gong, Lee, Lee, et al., 2006). When the levels of cholesterol decline, SCAP/SREBP detaches from INSIG1 and exits the ER. As a result, INSIG1 is rapidly ubiquitinated and degraded through the action of gp78, a membrane bound ubiquitin ligase (J. N. Lee, Song, DeBose-Boyd, & Ye, 2006). Thus, to block the activation of SREBPs, cells need to replenish their supply of both sterols and INSIG1 and both these processes are dependent on the transcriptional activity of SREBPs (Joseph L. Goldstein et al., 2006; Gong, Lee, Lee, et al., 2006). This type of regulation, which has been termed convergent inhibition, makes the system less dependent on cholesterol and ensures that active SREBP molecules have entered the nucleus and turned on their target genes.

Cholesterol metabolism is also regulated at the post-transcriptional level by various mechanisms including the degradation and phosphorylation of HMGCR, proprotein convertase subtilisin/kexin type 9 (PCSK9) dependent degradation of the LDL-R and the inducible degrader of the LDL receptor (IDOL) dependent ubiquitination of the LDL receptor. The HMGCR protein displays a long half-life, thereby maximizing mevalonate production for sterol and isoprenoid synthesis (Joseph L. Goldstein & Brown, 1990). Products from both pathways (sterols and isoprenoids) feedback to control HMGCR activity including the degradation of the enzyme (Joseph L. Goldstein et al., 2006). Although it has been known for some time that products of the mevalonate pathway control, a key discovery was the finding that the sterol intermediates, lanosterol and 24,25-dihydrolanosterol, rather than the end product cholesterol, stimulate HMGCR degradation (Song, Javitt, & DeBose-Boyd, 2005). Lanosterol is the first sterol synthesized from mevalonate flux into the sterol pathway and its accumulation stimulates binding of the HMGCR to INSIG, resulting in the ubiquitination of HMGCR on two cytosolic lysine residues and subsequent degradation by the 26S proteasome. In addition to lanosterol, oxysterols promote the ubiquitination and degradation of the HMGCR (Song & DeBose-Boyd, 2004). Although it remains to be determined, oxysterols are presumably acting through their ability to bind INSIG and to stimulate INSIG binding to HMGCR. High concentrations of mevalonate are required for the rapid degradation of HMGCR. In addition to lanosterol, the complete and rapid degradation of HMGCR requires additional non-sterol isoprenoid signaling (Sever et al., 2003a). The requirement for this signal can be filled by addition of geranylgeraniol, a 20-C isoprenyl alcohol, but not the 15-C alcohol farnesol (Sever et al., 2003b). It is not known, however, which geranylgeraniol-derived metabolite is acting to control the HMGCR degradation. In addition to the degradation of HMGCR, it has recently been shown that cholesterol accelerates the proteosomal degradation of SM, the enzyme that catalyzes the first oxygenation step in cholesterol synthesis (Gill, Stevenson, Kristiana, & Brown, 2011). Unlike HMGCR, SM degradation is not mediated by INSIG, 24,25-dihydrolanosterol, or side-chain oxysterols, but rather by cholesterol itself. This finding suggests an additional control point in cholesterol metabolism beyond HMGCR.

In addition, two additional players involved in the regulation of SREBP have recently been identified, erlins-1 and -2. Erlins-1 and -2 interact with the INSIG/SCAP/SREBP complex in fact, cholesterol directly binds to erlins-1 and -2. Huber et al. demonstrated that siRNA knockdown of erlins-1 and -2 continued to activate the SREBP pathway even in conditions of sterol excess, suggesting they play a role in restricting SREBP activation. Moreover, Insig, Scap, SREBP1a and SREBP-2 all co-immunoprecipitated with erlin-2 and the associated erlin-1 when

cellular cholesterol levels were high, but not when they were depleted, with INSIG being the more proximal binding partner. Together, these data support the hypothesis that *erlins* -1 and -2 play an important role in the cholesterol-dependent ER retention of SCAP/SREBP in cholesterol-sufficient conditions, possibly acting as scaffolds for the INSIG/SCAP/SREBP complex to retain SREBP in the ER or help in the formation of the necessary ER subdomain required to form the INSIG/SCAP/SREBP complex and/or promote the degradation of INSIG (Huber, Vesely, Datta, & Gerace, 2013).

3.2.4.3. Cholesterol uptaking regulation

From the cholesterol absorption point of view, any factor that changes the transportation of cholesterol from the intestinal lumen to the lymph can influence the efficiency of cholesterol absorption because intestinal cholesterol absorption is a multistep process. When dietary conditions are controlled, biliary factors may exert a major influence on the efficiency of cholesterol absorption, any changes in which may partly explain interindividual and interstrain differences in cholesterol absorption efficiency in humans and other animals (D. Q.-H. Wang, 2007). For example, hepatic output and pool size of biliary bile acids are markedly reduced in mice with homozygous disruption of the cholesterol 7 α -hydroxylase (CYP7A1) gene that encodes the key enzyme of the neutral pathway of bile acid synthesis (Schwarz, Russell, Dietschy, & Turley, 2001). As a result, the mice absorb only trace amounts of cholesterol because of bile acid deficiency in bile. Similarly, upon deletion of the sterol 27- hydroxylase (CYP27) gene, which encodes the main enzyme of the alternative pathway of bile acid synthesis, the knockout mice display significantly reduced bile acid synthesis and pool size. Consequently, intestinal cholesterol absorption decreases from 54% to 4%, whereas fecal neutral sterol excretion increases 2.5-fold (J J Repa, Lund, et al., 2000). However, in both knockout strains, cholesterol absorption is restored by feeding a diet containing cholic acid (J J Repa, Lund, et al., 2000; Schwarz et al., 2001). The absorption of biliary cholesterol is affected by its physical chemistry, but it is quite unclear whether dietary cholesterol is absorbed to the same degree as biliary cholesterol (van der Wulp et al., 2013).

At cellular level, PCSK9 enhances the degradation of the LDL-R (Benjannet et al., 2004). A large number of genetic variants of PCSK9 that may modulate plasma cholesterol levels have been found either positively or negatively. The PCSK9 gene is 25-kb long and comprises 12 exons and 11 introns. It encodes a 692-amino acid serine protease formerly called neural apoptosis regulated convertase-1 (NARC1), which is a member of the proprotein convertase

family of enzymes (Maxwell & Breslow, 2004). These convertases act as molecular scissors for tissue-specific processing of multiple precursor proteins. The catalytic domain of PCSK9 contains the main binding structure of PCSK9 to the epidermal growth factor-like repeat domain (EGF-A) of the LDL-R. The major function of the heterodimeric pro-segment PCSK9 is to degrade the LDL-R intracellularly and extracellularly, thereby acting as a chaperone that binds to the LDL-R to promote its lysosomal degradation. The deletion of PCSK9 protein results in an increase in LDL-R and a significant lowering of LDL cholesterol. Notably, recent studies have reported that PCSK9 inhibition using antibodies reduces LDL cholesterol over 60% (Chan et al., 2009).

3.2.4.4. Cholesterol efflux

Cholesterol removal is necessary for cholesterol homeostasis and serves to prevent cholesterol over-accumulation in cells. Because cells cannot degrade cholesterol, excess cholesterol must be removed and transported to the liver for reutilization and excretion in a process that is traditionally referred to as reverse cholesterol transport (RCT) (Glomset & Norum, 1973). HDL serves as the major acceptor for cellular cholesterol released from extrahepatic tissues, and is inversely correlated with the risk of atherosclerotic cardiovascular disease (Marina Cuchel & Rader, 2006). Excess cellular cholesterol is eliminated by diffusion mediated or apolipoprotein-mediated mechanisms (Oram & Yokoyama, 1996; Yokoyama, 2000). During passive diffusion, the removal of cholesterol is mediated by the exchange of cholesterol between the cell membrane and HDL. Many factors are likely involved in this process, such as cholesterol compartments in the plasma membrane and HDL structure. The enzyme lecithin-cholesterol acyltransferase (LCAT) has been suggested to also participate in this process (Yokoyama, 2000). LCAT is found on the surface of lipoproteins such as HDL and converts free cholesterol into cholesterol esters by transferring fatty acids from phosphatidylcholine to unesterified cholesterol (Norum, 2017). The cholesterol esters are then sequestered into the core of the lipoprotein and transported to the liver and steroidogenic tissues where they are selectively removed by the scavenger receptor class B type 1 (SRB1) (Ji et al., 1997). A large amount of CEs that are formed within HDL are also transferred to the triglyceride rich lipoprotein by the cholesteryl ester transfer protein (CETP). The remnants of this protein are converted to LDL and removed by the LDL receptor pathway or directly removed in the liver for excretion into bile (Linsel-Nitschke & Tall, 2005). Two ATP-binding cassette transporters, ATPbinding cassette subfamily G member 5 (ABCG5) and ATP-

binding cassette subfamily G member 8 (ABCG8), are half-transporters that function as heterodimers to mediate the excretion of cholesterol into bile (Berge et al., 2000). Mutations in either gene lead to sitosterolemia, a recessive disease that is characterized by increased cholesterol absorption and impaired biliary secretion (Yoo, 2016).

Another mechanism for cholesterol removal is mediated mostly by apoA1 (the major apoprotein of HDL) and leads to the assembly of discoidal HDL along with phospholipids and cholesterol (S. Wang & Smith, 2014). ABCA1 initiates HDL formation in the liver and is the first step in RCT (Tall, Yvan-Charvet, Terasaka, Pagler, & Wang, 2008). Several mechanisms have been proposed to facilitate ABCA1-mediated cholesterol efflux to lipid-poor apoA1. In one mechanism, apoA1 forms complexes with phospholipids and cholesterol at the cell surface, and is subsequently internalized and targeted to intracellular compartments, while lipidation of apoA1 occurs as part of the retroendocytosis pathway (Neufeld et al., 2004). In another mechanism, apoA1 forms complexes with phospholipids and cholesterol at the plasma membrane, this process is also mediated by ABCA1 (Nandi et al., 2009). The last mechanism is based in a small pool of apoA1 binds to ABCA1, enhancing net phospholipid translocation and thus, membrane strain (Vedhachalam et al., 2007). The membrane strain is relieved by the bending and creation of exovesiculated lipid domains, which promotes the binding of apoA1. In addition to apoA1, this lipid transport pathway has broad specificity for multiple exchangeable apolipoproteins including apolipoprotein A2, apolipoprotein E, apolipoprotein C and apolipoprotein A4 (Remaley et al., 2001). While ABCA1 is the predominant factor needed for cholesterol efflux to apoA1 and the formation of pre-b HDL, other factors, such as LCAT, and the ABC transporters, ABCG1 and ATP-binding cassette subfamily G member 4 (ABCG4), are needed for the maturation of HDL (van der Velde, 2010). After pre-b HDL is formed it must undergo further lipidation. Moreover ABCA1 and ABCG1 and ABCG1/ ABCG4 heterodimers synergistically mediate cholesterol efflux to HDL (Gelissen et al., 2006), however, the mechanism by which these transporters promote cholesterol efflux remains to be determined.

One of the main regulators of the cholesterol efflux are the Liver X Receptor proteins (LXRs) which also contribute to cholesterol homeostasis. The LXRs, LXR α and LXR β , are nuclear receptors that form heterodimers with retinoid X receptors (RXRs) and are activated by a variety of sterol metabolites (Willy et al., 1995). Whereas LXR β is expressed ubiquitously, LXR α is primarily expressed in the liver, adipose tissue, and macrophages and thus plays an important role in lipid metabolism (Joyce J. Repa & Mangelsdorf, 2000). LXRs activate the transcription of genes involved in cholesterol efflux, including ABCA1, ABCG1, and ABCG5/8.

When cholesterol levels surpass the biosynthetic rate, a feed-forward pathway is initiated that leads to the clearance of cholesterol. The binding of oxysterols to LXRs triggers a conformational change in the receptor that enhances interaction with co-activator proteins, thereby facilitating transcription of the aforementioned target genes and RCT (Tontonoz & Mangelsdorf, 2003). In vivo studies show that LXR-deficient mice accumulate sterols in their tissues and develop accelerated atherosclerosis (Bradley et al., 2007), whereas synthetic LXR agonists stimulate ABCA1 expression and reverse cholesterol transport (J J Repa, Turley, et al., 2000).

3.2.4.5. Other cholesterol regulation mechanisms

In addition to classic regulatory mechanisms, miRNAs have also been shown to regulate the expression of key genes in cholesterol metabolism. miRNAs are small (~ 22 nt), single-stranded, non-coding RNAs that regulate gene expression post-transcriptionally.

In the cholesterol regulation mechanisms have been identified *miR-33a* and *miR-33b*, intronic miRNAs located within the *Srebp-2* and *Srebp-1* genes, respectively (Rayner et al., 2010). Metabolic stimuli that activate the expression of *Srebp-2* and *Srebp-1* lead to an increased expression of *miR-33a* and *miR-33b*, respectively, suggesting that both host genes and miRNAs are co-regulated. *miR-33a* targets genes involved in cholesterol trafficking, including ABCA1, ABCG1, and NPC1 (Gerin et al., 2010). Interestingly, ABCA1, a transporter responsible for the movement of cholesterol out of the cell, was among the top predicted target genes for *miR-33a*. *miR-33a* overexpression strongly represses ABCA1 expression and decreases cellular cholesterol efflux to apoA1. On the other hand, antagonism of endogenous *miR-33* up-regulates ABCA1 expression in vitro and in vivo, and promotes cholesterol efflux to apoA1, further confirming the physiological effects of *miR-33* (Najafi-Shoushtari et al., 2010). Together, these findings establish a reciprocal pathway in which, during sterol-poor conditions, *miR-33a* is coincidentally generated with SREBP2 and works to increase cellular cholesterol levels by down-regulating ABCA1 and ABCG1 and thus, limit cholesterol efflux. As mentioned above, ABCA1 plays a key role in regulating HDL biogenesis in vivo. Remarkably, antagonists of *miR-33* in vivo using locked nucleic acid modified oligonucleotides, lentivirus, and adenovirus increase significantly the expression of ABCA1 in the liver and plasma HDL levels (Horie et al., 2010).

Finally, IDOL is an E3 ubiquitin ligase (transcriptionally activated by LXR agonists) that triggers ubiquitination of the LDL-R on its cytoplasmatic domain, thereby targeting it for

degradation. Unlike the LDL-R and *Pcsk9* genes, *Idol* is not regulated by SREBPs. IDOL knockdown in hepatocytes increases LDL-R protein levels and promotes LDL uptake (Zelcer, Hong, Boyadjian, & Tontonoz, 2009). Conversely, adenovirus-mediated expression of IDOL in mouse liver promotes LDL-R degradation and elevates plasma LDL levels. Interestingly, IDOL targets two closely related LDL-R family members, VLDLR and apoE receptor 2 (ApoER2), two proteins implicated in both neuronal development and lipid metabolism. IDOL triggers ubiquitination of the VLDLR and ApoER2 on their cytoplasmic tails, leading to their degradation (Hong et al., 2010).

3.3. Atherosclerosis

Related with the overaccumulation of cholesterol (**Table 3**) are cardiovascular diseases (CVD) which lead the cause of mortality worldwide, accounting for 16.7 million deaths each year (Thomas et al., 2018), about one third of total global deaths. Atherosclerosis, an inflammatory disorder of the vasculature, is the primary cause of CVD-related events, including myocardial infarction and stroke (Badimón, Vilahur, & Padró, 2009). Given the increase in prevalence of obesity and diabetes in developing countries, the global incidence of CVD is predicted to increase and impose a greater economic burden on the health-care services around the world. Under normal healthy conditions, the metabolism and transport of cholesterol, including influx and efflux within cells, is highly regulated. The development of atherosclerosis (**Figure 6**) can begin when these homeostatic mechanisms become unbalanced in favor of either increased cholesterol influx or decreased efflux. Within the blood, there are several lipoproteins that each has a different function in lipid transportation.

LDL is one of the most important lipoproteins found in the bloodstream and its function is to transport cholesterol from the liver to the peripheral tissues (McLaren, Michael, Ashlin, & Ramji, 2011). In order to maintain a balance in cholesterol metabolism, HDL transports excess cholesterol from the peripheral tissues back to the liver for excretion via the bile system by a process known as reverse cholesterol transport. However, only 5% of the biliary cholesterol is excreted in feces as the rest is reabsorbed in the intestine (McLaren et al., 2011). Given that high LDL-cholesterol and low HDL-cholesterol levels have been associated with reduced endothelial function, increased LDL-cholesterol and HDL-cholesterol levels are thought to be pro-atherogenic and anti-atherogenic, respectively (Vogel, 1997). Therefore, strategies for treating atherosclerosis should be aimed at lowering plasma LDL levels and increasing serum HDL levels.

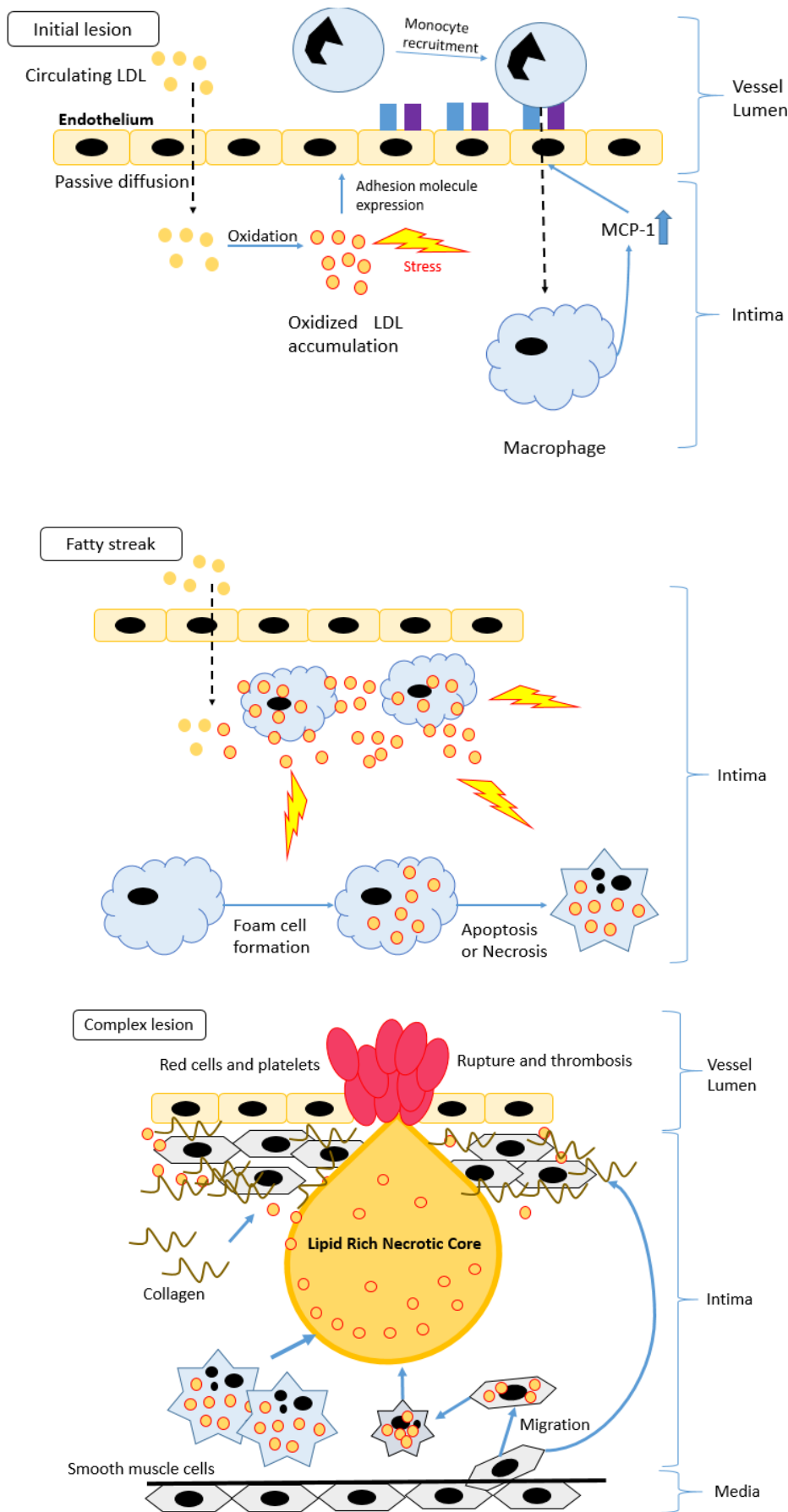


Figure 6. The development of atherosclerosis. The expression of pro-inflammatory genes, including ICAM-1 and MCP-1, is triggered by the build-up of modified LDL in the neighbor endothelial cells during the development of the **initial lesion**. Circulating monocytes are then recruited to the modified LDL accumulation and migrate into the intima and differentiate into macrophages. Once in the walls of the artery, the macrophages are able to take up the modified LDL and become lipid-laden foam cells, which can accumulate and form a **fatty streak**. During complex lesion formation, foam cell lysis by apoptosis and necrosis leads to the formation of a necrotic core, and together with defective efferocytosis, leads to the amplification of the inflammatory response. SMCs begin to migrate from the media to the intima and the ECM produced by them forms fibrous cap and stabilizes the plaque. SMCs also transform to foam cells. During later stages of the **complex lesion** the plaque can become unstable owing to the inflammatory response, resulting in an inhibition of ECM formation, particularly collagen production by SMCs. The remaining ECM can then start to be degraded by proteases released by macrophages, resulting in an unstable lesion that can rupture and lead to thrombosis. These events can cause a myocardial infarction or stroke, depending on the location of plaque formation.

ECM, Extracellular matrix; ICAM-1, Inter cellular adhesion molecule-1; LDL, Low density lipoprotein; MCP-1, Macrophage chemoattractant protein-1; SMCs, Smooth muscle cells.

The first stage of atherosclerosis is the internalization of cholesterol via circulating LDL in the intima, with the concomitant endothelial activation/ dysfunction. The vascular endothelium is a semipermeable barrier that controls the diffusion of plasma molecules and regulates vascular tone, inflammation and prevents thrombus formation (Komarova, Kruse, Mehta, & Malik, 2017), properties that are lost with the dysfunctional endothelium. Consequently, LDL particles further infiltrate and accumulate in the extracellular matrix (ECM); circulating monocytes are then recruited and attached to the vascular endothelium due to the exposure to endothelial adhesion molecules. Once attached, they transmigrate into the sub endothelial space where they are transformed into macrophages. Moreover, injury in the endothelial-related antithrombotic properties facilitates platelet adhesion and activation in the dysfunctional area. Adhered platelets, in arrangement with dysfunctional endothelial cells, secrete chemotactic and growth factors, which stimulate migration, accumulation and proliferation of vascular smooth muscle cells (VSMC) and leukocytes in the intimal layer, promoting plaque progression (Jennings, 2009).

LDLs retained in the ECM mainly by proteoglycans become targets for oxidative and enzymatic modifications. Oxidized LDLs (oxLDLs) enhance a series of pro-inflammatory reactions via different mediators perpetuating the activation, recruitment and transmigration of monocytes and other inflammatory cells across the endothelial layer into the intima. In addition, the attracted macrophages scavenge oxLDLs, become lipid laden, and convert into foam cells. In the early steps of atherosclerosis, accumulation of foam cells evolves into fatty streaks. Complication of the lesion occurs when foam cells release growth factors and cytokines, which further stimulate VSMC migration from the media into the intima where they divide and produce ECM components such as collagen and contribute to the formation of a fibrous cap.

If the pathological process persists and macrophages fail to remove accumulated cholesterol from the vessel, they become apoptotic, releasing cholesterol to the vessel wall and, more importantly, pro-thrombotic molecules and metalloproteinases (Ghosh, Zhao, Bie, & Song, 2010). Progression and complication of atherosclerotic plaques are also characterized by a decreased number of VSMCs as well as the formation of immature and leaky new vessels, making atherosclerotic lesions more susceptible to rupture. Plaque disruption and the subsequent exposure of thrombogenic substrates initiate both platelet adhesion/activation and aggregation on the exposed vascular surface and the activation of the coagulation cascade, leading to thrombus formation and clinical manifestations of the atherosclerotic

disease, acute myocardial infarction or sudden death (Legein, Temmerman, Biessen, & Lutgens, 2013).

A large part of the anti-atherogenic and anti-thrombotic properties of the vascular endothelium are mediated by its capacity to produce and release substances such as nitric oxide (NO), a platelet aggregation inhibitor with a strong vasodilatory activity and an important antiinflammatory function. NO blocks the expression of proinflammatory molecules such as necrosis factor kappa B (NF- κ B) and adhesion molecules such as intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) as well as leukocyte infiltration and adhesion. Tight and gap junctions are cell-cell connections with essential structures in regulating the endothelial permeability function. The formation of gap junctions is regulated by the presence and functionality of connexins, proteins whose expression is altered during the formation of atherosclerotic lesions. Gap junctions favor intercellular signaling processes and regulate NO-dependent vasodilation (Badimón et al., 2009). Atherogenic concentrations of LDL lead to a decrease in the bioavailability of endothelial NO. This decrease in NO availability is associated with a reduction in the concentration and/or activation of the endothelial NO synthase (eNOS), as a result of the presence of native LDL or modified LDL particles, as well as the degradation of NO through the formation of superoxide anions (O_2^-). The imbalance between the redox state and NO is associated with protein nitrosylation. Deactivation of NO by O_2^- gives rise to highly cytotoxic peroxynitrite radicals. The increase of peroxynitrites derived from nitrotyrosines and the production of O_2^- are characteristics present in human atherosclerotic lesions (Pritchard et al., 1995).

It has been demonstrated that increased formation of reactive oxygen species (ROS) and/or altered oxygen utilization contributes to atherogenesis by superoxide production that mediates endothelial dysfunction and increases oxLDL levels. Small oxidized lipids that are components of oxLDL, such as oxysterols, oxidized fatty acids and aldehydes, are potent inducers of ROS production (Littlewood & Bennett, 2003). ROS in the vascular wall are generated by enzymes such as NADPH oxidase, xanthine oxidase and eNOS. Formation of intracellular ROS in the mitochondrial electron transport chain is controlled by antioxidant mechanisms. It has been shown that the increase of ROS generation by the mitochondria triggers cytochrome C release leading to caspase activation and apoptosis. The generation of large amounts of ROS can overwhelm the intracellular antioxidant defense, causing activation of neutrophils, protein modification, lipid peroxidation, and DNA damage, key factors for the initiation of atherosclerosis and the development of CVD.

Although excess cholesterol in blood is related with atherosclerosis, the exact causes and risk factors are unknown; however, certain conditions, traits, or habits may raise the chance of developing atherosclerosis. Most risk factors including high total cholesterol and LDL cholesterol (LDL-C), low level of HDL in blood, hypertension, tobacco smoke, diabetes mellitus, obesity and sedentary lifestyle can be controlled and atherosclerosis can be delayed or prevented. Across cultures there are many different dietary lifestyles, some of which promote health and others that increase risk of atherosclerosis (Torres, Guevara-Cruz, Velázquez-Villegas, & Tovar, 2015).

3.4. Familial hypercholesterolemia

Familial hypercholesterolemia (FH) is among the commonest inherited metabolic disorders. It is caused due to a group of genetic disorders that result in abnormally high LDL cholesterol (LDL-C) levels that cause premature atherosclerotic plaque deposition in arteries and a markedly increased risk of CVD at a young age. The inheritance was first described in the 1960s with an autosomal codominant inheritance with homozygotes having twice the LDL-C levels of heterozygotes due to the abnormal metabolism of LDL (FREDRICKSON & LEES, 1965; KHACHADURIAN, 1964).

3.4.1 Prevalence

The frequency of the heterozygous FH state has been estimated at 1 in 500 and of the homozygous FH state at 1 in 1.000.000 (M. Cuchel et al., 2014). Initial prevalence estimates for heterozygous FH were based on the relatives of the survivors of myocardial infarction (J L Goldstein, Schrott, Hazzard, Bierman, & Motulsky, 1973). Higher heterozygous frequencies ranging from 1:67 to nearly 1:400 have been reported in certain populations. In these population groups, it is hypothesized that genetic drift led to the expansion of genotypes established by a few probands (founder effect) (Bétard et al., 1992). The recent investigation of an unselected community-based population of nearly 69.000 individuals in Denmark has suggested that the prevalence of heterozygous definite or suspected FH may be as high as 1 in 137 (Benn, Watts, Tybjaerg-Hansen, & Nordestgaard, 2012). It follows that the prevalence of homozygous FH may be as high as 1 in 160.000 in this population. It is important to remember that most prevalence estimates cited in the literature are based on clinical and not genetic criteria. Patients with FH predominantly have an excess of CVD rather than cerebral or peripheral arterial disease. The risk of premature CVD is elevated to about 20-fold in heterozygous FH, with the highest risk being noted in young untreated men (Watts, Lewis, & Sullivan, 2007) . Patients with homozygous FH typically develop CVD by the second decade of life. CHD deaths in the first decade of life have been reported as well. Cases of valvular and supra-avalvular aortic stenosis due to lipid deposition have been reported with homozygous FH and rarely with heterozygous FH (Ozumi et al., 2005). Systematic data evaluating the prevalence of FH in most countries/territories are lacking. The majority of patients with FH are either untreated or undertreated (K. K. Ray et al., 2014).

3.4.2 Genetics

In most cases, FH has dominant or co-dominant inheritance with over 90 % penetrance (J L Goldstein & Brown, 1979). Mutations in LDL-R, apoB, LDLRAP1, and PCSK9 genes between others have been linked to FH (**Table 2**). Known variants in these genes have been found in over 80 % of patients with FH. Heterozygous FH is caused by a pathogenic variant in one allele. In contrast, homozygous FH results from either biallelic mutations in one of the known genes (true homozygotes) or compound heterozygosity for two different mutations in the same or different candidate genes known to cause FH.

Mutations in LDL-R were identified as a cause of FH in the 1970s and are seen in >80 % of patients with FH (J L Goldstein & Brown, 1974b). The gene for LDL-R lies on the short arm of chromosome 19 (19p13.1– 13.3). Defects noted in the LDL-R can be classified into five broad categories: defective ligand binding, defective transport, defective internalization, recycling, and complete lack of receptors (Soutar & Naoumova, 2007). At present, >1600 mutations have been identified that can be consulted in the University College London Low Density Lipoprotein Familial Hypercholesterolemia Database. The pathophysiology of the LDL receptor is the following:

1. Decreased LDL receptor function due to a genetic defect, typically one of the following classes:
 - LDL-R is not synthesized.
 - LDL-R is not properly transported from the ER to the Golgi apparatus for expression on the cell surface.
 - LDL-R does not properly bind LDL on the cell surface.
 - LDL-R does not properly cluster in clathrin-coated pits for receptor endocytosis.
 - LDL-R is not recycled back to the cell surface.
2. LDL-R -mediated endocytosis is decreased.
3. Leading to markedly elevated LDL levels
4. Premature development of atherosclerotic plaque.

Table 2. Known mutations in familial hypercholesterolemia.

Mutation	Gene	Mechanism	Number of mutations (% of FH cases)	Inheritance
LDL receptor	LDL-R	LDL receptor is absent or has decreased capacity to clear LDL from circulation	>1600 (85-90%)	Autosomal Co-Dominant
ApoB (familial defective apoB)	ApoB	Impaired LDL receptor binding- mutation at binding site on LDL particle	32 Most common is Arg3500Gln (5-10%)	Autosomal Co-Dominant
PCSK9 gain of function	PCSK9	Increased PCSK9 level leads to increased degradation of LDL receptors	23 Rare	Autosomal Co-Dominant
Apolipoprotein E	ApoE	Impaired LDL receptor binding- mutation at binding site on LDL particle	1 Very rare	Autosomal Dominant
Signal-transducing adaptor protein 1	STAP1	Impaired LDL receptor signaling	4 Very rare	Autosomal Dominant
Sterolin 1/ 2	ABCG5 / ABCG8	Impaired cholesterol efflux	2 Very Rare	Recessive
LDL receptor adaptor protein	LDLRAP1	Protein needed for clathrin-mediated internalization of LDL receptor	17 Rare	Recessive
Lysosomal acid lipase	LIPA	Unable to process extracellular cholesterol	1 Very Rare	Recessive

apoB is present on the LDL particle surface and serves as the ligand for the LDL-R. Mutations in apoB (chromosome 2p23–24) have been identified in about 2–5 % of cases in northern Europe but have not been commonly noted in other populations. The APOB mutation that has been most commonly detected among northern Europeans is Arg3500Gln (haplotype inherited from a common ancestor 6000–7000 years ago) (Myant, Forbes, Day, & Gallagher, 1997). Another substitution at the same codon-Arg3500Trp has been noted in the Chinese population (Tai, Pan, & Lee-Chen, 1998). Apo B mutations have been reported as having variable penetrance.

PCSK9 spans codes for a protein that serves as a post-transcription inhibitor of LDL-R by cell-surface interaction (to induce LDL-R degradation). Mutations leading to “gain” of function of PCSK9 activity account for < double heterozygote < homozygous APOB or PCSK9 gain-of-function mutation < homozygous LDLRAP1 or LDL-R defective mutations < compound heterozygote: LDL-R defective plus LDL-R-negative (null) mutation < homozygous LDL-R-negative (null) mutation (M. Cuchel et al., 2014).

Very rarely, mutations in other genes involved in lipoprotein metabolism can cause FH. DNA sequencing efforts in individuals with FH in whom no mutations in LDL-R, ApoB or PCSK9 could be detected found dominant mutations in ApoE (apolipoprotein E) and STAP1 (signal-transducing adaptor protein 1), but these cases remain exceedingly rare (Awan et al., 2013; Fouchier et al., 2014). In a few families in which the severe FH phenotype segregates as a recessive trait, genetic mapping studies have identified causative homozygous mutations in LDLRAP1 (Fellin, Arca, Zuliani, Calandra, & Bertolini, 2015). Individuals with LDLRAP1 mutations are diagnosed clinically with severe hypercholesterolemia and usually also receive a genetic diagnosis of homozygous FH. Their phenotype is generally less severe than that of patients with homozygous FH due to LDL-R mutations (M. Cuchel et al., 2014). Next-generation DNA sequencing analyses of patients with severe hypercholesterolemia and apparently recessive inheritance of this phenotype have also identified rare mutations on both alleles of other genes that have been associated with dyslipidaemia, namely, ABCG5, ABCG8 and lysosomal acid lipase (LIPA) (Stitzel et al., 2013).

Mutations in ABCG5 or ABCG8 can cause sitosterolemia (an extremely rare condition in which plant sterols accumulate in the blood and tissues) and mutations in LIPA can cause lysosomal acid lipase deficiency (cholesterol ester storage disease or Wolman disease) (Fouchier & Defesche, 2013; Patel, 2014). Such findings suggest that mutations in genes that have been linked to other syndromic lipid disorders can also rarely cause high LDL cholesterol

levels and should be considered in individuals with apparent FH but without mutations in the most common causative genes.

Occasionally, patients carry more than two mutations in genes associated with FH (M. Cuchel et al., 2014). However, depending on the LDL-C threshold used for diagnosis, in at least 20–40% of patients with possible or probable heterozygous FH by clinical criteria, no causative mutation in any gene associated with FH (Talmud et al., 2013). Although it remains possible that these patients might carry a mutation in a gene known to cause FH but that the tests did not detect the mutation or — less probably — that a mutation occurred in a new causative gene, a large proportion of these patients instead have a severe form of polygenic hypercholesterolemia (Talmud et al., 2013).

In the general population, there are numerous common single nucleotide polymorphisms (SNPs) in a variety of genes that have been associated with LDL-C levels in large-scale genome-wide association studies (Willer et al., 2013). Each of these SNP *loci* slightly raises or decreases LDL-C by an incremental amount. The distribution of these independently segregating small-effect SNP alleles is such that most individuals carry a balance of LDL cholesterol-raising and LDL cholesterol-lowering alleles. However, individuals at the high extreme of this distribution have inherited a preponderance of LDL cholesterol-raising alleles that cumulatively can raise LDL cholesterol into the range observed in patients with heterozygous FH due to large-effect single-gene mutations (Willer et al., 2013). At present, there is no clinical standard for which precise loci and alleles should be included in a genetic risk score (Jian Wang et al., 2016). Clinically, if these patients have elevated LDL-C and a family history of dyslipidemia or CVD, for all practical purposes they can be diagnosed with FH. SNPs and polygenic contribution to lipid levels may also mostly account for variable penetrance noted in families, besides environmental factors, dietary influences, gene-environment interactions, epigenetic influences, and random variation (Ferrières, Lambert, Lussier-Cacan, & Davignon, 1995). This may also explain milder phenotypes rarely seen with severe mutations. For example, LDL-C levels <500mg/dl have been reported in homozygous FH.

3.4.3. *Diagnosis*

Although the atherosclerotic manifestations of FH usually occur in adulthood, the clinical effects of the disease can start in the first decade of life in homozygous patients (M. Cuchel et al., 2014). Unfortunately, FH is often diagnosed late and after the occurrence of a major coronary event. A combination of screening methods to identify at risk individuals is needed to prevent premature atherosclerosis (Goldberg et al., 2011).

Patients with heterozygous FH are generally asymptomatic in childhood and early adulthood. About 5% of heart attacks under the age 60 and as many as 20% under age 45 are due to FH (Hopkins, Toth, Ballantyne, Rader, & National Lipid Association Expert Panel on Familial Hypercholesterolemia, 2011). Homozygous or compound heterozygous FH has a severe and variable clinical presentation usually within the first decade of life. Most of these individuals have extreme hypercholesterolemia with rapidly accelerated atherosclerosis when left untreated. The variation depends of the amount of LDL-R activity (Raal & Santos, 2012a). Coronary artery disease is the most common cause of premature death in these patients, but other cardiovascular disease including aortic and supraaortic stenosis and aortic root disease is also common.

There are a number of barriers to the diagnosis and treatment of FH. Many individuals and family members with FH who have CVD have other common CVD risk factors; thus genetic hypercholesterolemia is not suspected and ultimately not diagnosed. Primary care physicians manage most patients with hypercholesterolemia, and there is often a lack of awareness of FH among physicians and the general public with only very severe cases being referred to specialists (Watts et al., 2014).

Cascade screening, in which health care providers actively screen for disease among the first and second degree relatives of patients diagnosed with FH, can increase detection rates but risks missing affected individuals. There are guidelines recommending universal screening for elevated serum cholesterol by age 20 and cascade testing of first-degree relatives of all individuals with FH (Watts et al., 2011). For children, cholesterol screening should be done at age 9 to 11 and considered beginning at age 2 in those with a family history of premature cardiovascular disease or elevated cholesterol (Daniels, Gidding, de Ferranti, & National Lipid Association Expert Panel on Familial Hypercholesterolemia, 2011).

Overall, diagnosis of FH is based on lipid levels (**Table 3**), family history, physical findings (if present), and genetic analysis. Physical examination findings of tendon xanthomas, arcus corneae (under age 45) and tuberous xanthomas or xanthelasma (under age 25) when

present at an early age should also prompt suspicion for FH. However, physical findings are not present in all patients with FH (Hopkins et al., 2011). Taken together, the general clinical approach to consider FH is the following:

1. Presence of premature atherosclerotic cardiovascular disease
2. Fasting LDL-C levels >190 mg/dL in adults after exclusion of secondary causes of elevated LDL-C (hypothyroidism, nephrotic syndrome).
3. Fasting untreated LDL-C levels that have an 80% probability of FH in the general population:
 - ≥ 250 mg/dL in adults ≥ 30 years.
 - ≥ 220 mg/dL in adults aged 20 to 29.
 - ≥ 190 mg/dL in patients under age 20.
4. Presence of full corneal arcus under age 45.
5. Presence of tendon xanthomas.
6. Family history of premature atherosclerotic cardiovascular disease.
7. Family history of high cholesterol levels.

On the other hand, the diagnosis of homozygous (or compound heterozygous) FH has been defined in a number of ways (Raaijmakers & Santos, 2012a), with one possible definition in the next list:

1. Genetic analysis showing mutations in two alleles at gene locus for LDLR, APOB, PCSK9, LDLRAP1 or related.
2. Presence of untreated LDL >500 mg/dL or treated LDL > 300 mg/dL plus one of the next parameters:
 - Presence of cutaneous or tendon xanthomas before the age of 10 years.
 - Both parents with evidence of heterozygous FH (except for the rare LDLRAP1 mutations).

Clinical criteria may not identify all patients with FH, and genetic testing is part of screening strategies in a number of countries, with the costs covered by national health services (Nordestgaard et al., 2013). Genetic testing in certain populations has changed understanding of the frequency of both heterozygous and homozygous FH as mentioned previously. However, a mutation is not always found in patients with clinical FH, and lack of a mutation should not change treatment.

Table 3. Average blood cholesterol level recommendations. Based on the USA Cholesterol Guidelines. These data can vary depending on age and sex.

	Desirable (mg/dl)	Caution (mg/dl)	Dangerous (mg/dl)
Total Cholesterol	<200	200-239	>240
LDL-C	<130	130-159	>160
HDL-C	>50	40-49	<40
Triglycerides	<200	200-399	>400

3.4.4. Treatments

The lifetime risk of CVD and premature onset CVD is very high in individuals with FH. Early treatment is beneficial and long term drug therapy can substantially reduce or eliminate the added lifetime risk of CVD from having FH and can lower the CVD event rate in heterozygous FH patients to levels similar to those of the general population (Versmissen et al., 2008). However, the first steps to treat FH is by life style changes as follows:

1. Dietary modification contributes to improvement in lipid profiles:
 - A heart healthy diet including vegetables, fruit, non-fat dairy, beans, tree nuts, fish and lean meats should be encouraged.
 - Restrict intake of saturated fat to less than 7% of calories.
 - Avoid trans fatty acids.
 - If alcohol is used, amount should be moderate.
 - Addition of plant stanols and insoluble fiber can provide some LDL-C lowering.
 - Dietitian counseling.
2. Physical activity.
3. Avoidance of weight gain.
4. Avoidance and cessation of smoking is mandatory.
5. Discourage exposure to passive smoking.
6. Treat diabetes and hypertension.
7. Consider low-dose aspirin.

Homozygous patients require treatment as soon as the diagnosis is made and need lifestyle, medication and additional modalities. Treatment of homozygous FH patients can delay major cardiovascular events and early death (Raaijmakers et al., 2011).

3.4.4.1. Statins

The most effective oral agents for the prevention and treatment of cardiovascular diseases associated to dyslipidemia are the statins (**Figure 7**). Statins, inhibitors of the HMGCR, are molecules of fungal origin. By inhibiting a key step in the sterol biosynthetic pathway statins are powerful cholesterol lowering medications.

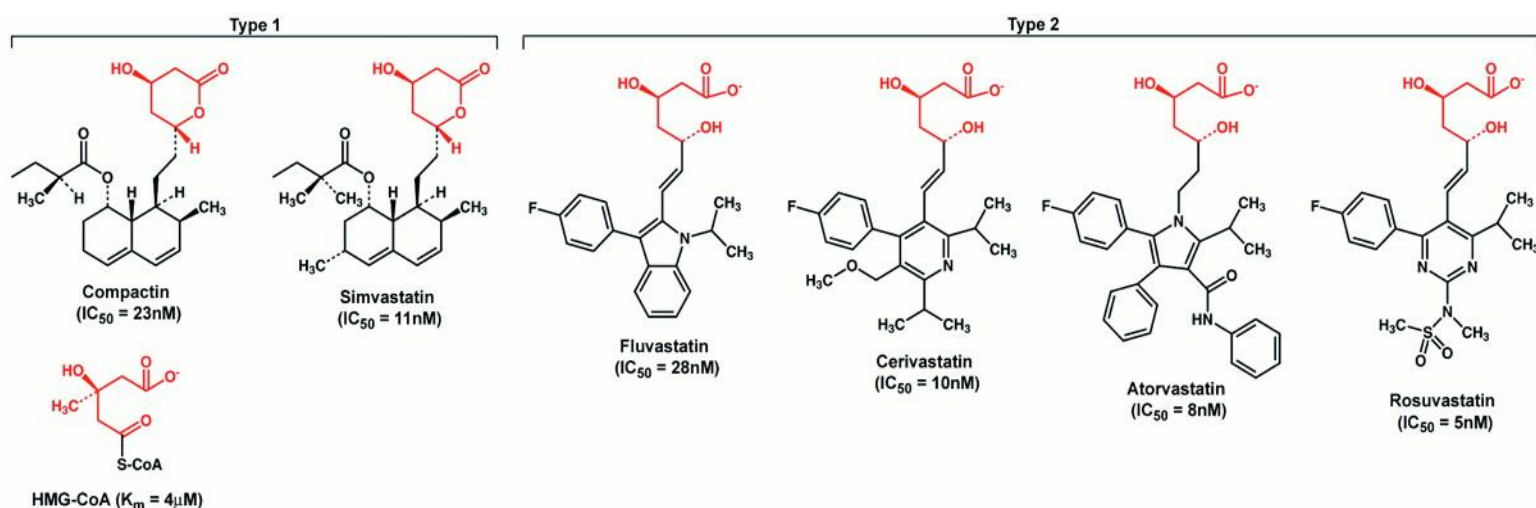


Figure 7. Structural formulas of statin inhibitors and the enzyme substrate HMG-CoA. Structure of several statin inhibitors. Compactin and simvastatin are examples of type 1 statins; not shown are the other type 1 statins, lovastatin and pravastatin. Fluvastatin, cerivastatin, atorvastatin, and rosuvastatin are type 2 statins. The HMG-like moiety that is conserved in all statins is colored in red. The IC_{50} (median inhibitory concentration) values of the statins are indicated. Structure of HMG-CoA. The HMG-moiety is colored in red, and the K_m value (Enzyme affinity) of HMG-CoA is indicated. Adapted from (Istvan & Deisenhofer, 2001).

HMG-CoA, 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A

All statins can reduce LDL cholesterol in FH, and the absolute reductions achieved in heterozygous FH often exceed those observed in the general population, because of the higher baseline LDL cholesterol in patients (Besseling, Hovingh, Huijgen, Kastelein, & Hutten, 2016). By contrast, the response to statins in patients with homozygous FH is attenuated compared with that of the general population (Robinson, Goldberg, & National Lipid Association Expert Panel on Familial Hypercholesterolemia, 2011). For the same reason, high-potency statins are preferred, owing to their superior efficacy. Statins reduce CVD risk in both homozygous and heterozygous FH (Raaijmakers et al., 2011). Statins are generally well tolerated by patients, although doses are higher than those usually prescribed (Cupido, Reeskamp, & Kastelein, 2017).

Statins should be the initial drug for all adults with FH and in children with heterozygous FH starting at 8 to 10 years of age (Ito, McGowan, Moriarty, & National Lipid Association

Expert Panel on Familial Hypercholesterolemia, 2011). Patients with homozygous FH should be treated as soon as the diagnosis is made (Raai & Santos, 2012b). The Food and Drug Administration (FDA) has approved lovastatin, atorvastatin, simvastatin and rosuvastatin children above 10 years of age and pravastatin in those over 8 years. Statins increase the expression of LDL receptors by reducing HMGCR, the rate-limiting step in cholesterol synthesis. Moderate to high potency statins should be used as first line treatment (atorvastatin, rosuvastatin, simvastatin, pitavastatin). Low potency statins are usually inadequate for FH patients (Watts et al., 2014). Adult FH patients should have a treatment goal of $\geq 50\%$ LDLC reduction from baseline. Statin therapy is effective in heterozygous FH patients and may also benefit homozygous patients who have some LDL receptor activity (M. Cuchel et al., 2014).

Long-term safety of statins in the pediatric population is still unknown, but the current benefits of therapy outweigh the risk of untreated pediatric populations (Daniels et al., 2011). Children and adolescents being treated with statins should have regular follow up with close monitoring of creatinine kinase (CK), aspartate amino transferase (AST) and alanine amino transferase (ALT) levels. Baseline levels, then repeat testing should be done at 1-3 months after drug initiation and then yearly. If CK levels reach five times and AST or ALT three times the upper limit of normal, a 3-month drug-free holiday should be initiated with reintroduction of the same drug at a lower dose or a different statin if levels return to baseline (Varghese, 2014). Additionally, high lipoprotein (a) (A similar form of LDL from which it differs due to the presence of apolipoprotein (a) bound to apoB via one disulfide bridge) levels were recently found to be an independent risk factor for CVD among asymptomatic statin-treated FH patients (Vuorio, Watts, & Kovanen, 2019).

Patients with FH who have a higher risk of CVD require more intensive drug therapy. High-risk patients include those with:

1. Clinically evident CVD.
2. Diabetes.
3. Family history of very early CVD (<45years in men and <55 years in women).
4. Current smoking.
5. Two or more CVD risk factors.
6. High lipoprotein (a) ($\geq 50\text{mg/dL}$).
7. In these patients, the LDL goal is $<100\text{ mg/dL}$ and non-HDL goal is $<130\text{ mg/dL}$.

3.4.4.1.1. Statins side effects

Approximately 25% of the world population older than 65 years take a statin for primary or secondary prevention of cardiovascular diseases (Gu, Paulose-Ram, Burt, & Kit, 2014). Although statins are generally well tolerated, patients treated with statins may complain of diminished lower extremity muscular strength (Loenneke & Loprinzi, 2018). Moreover, statins may produce statin-induced myotoxicity (SIM), including heterogeneous clinical manifestations such as muscle weakness, muscle pain or aching (myalgia), stiffness, muscle tenderness, cramps, and arthralgia. Any of these symptoms can be presented with or without an elevation of CK serum concentrations released from muscles in response to exercise, which can signal skeletal muscle injury in rare cases (Smit, Bär, Geerdink, & Erkelens, 1995). On the contrary, elevation of serum CK might be the only sign of SIM.

Statin intolerance occurs in 10–15% of patients with FH (Stroes et al., 2015). The management of statin intolerance in these patients is similar to that in other groups with high CVD risk and includes statin re-challenge (that is, the reintroduction of a statin after suspending the treatment for ≥ 4 weeks, during which time symptoms abate), switching to a different statin, reducing statin doses or increasing dosing intervals and use of combination treatment with non-statin agents (Mancini et al., 2016).

Also liver toxicity from statins is often a fact, it is not common, and serious hepatotoxicity is extremely rare. Hepatic aminotransferase elevation is usually mild and does not require discontinuation of the statin. It may be dose dependent. Only about 1% of patients have aminotransferase increases to greater than 3 times the upper limit of normal, and the elevation often decreases even if patients continue on the statin. A common cause is hepatic steatosis, which responds to weight loss. Statins can be used cautiously in the presence of liver disease as long as it is not decompensated (Herrick, Litvin, & Goldberg, 2014). In particular, nonalcoholic fatty liver disease is not a contraindication. Hepatic transaminases should be obtained at baseline and during treatment if there is a clinical indication for their measurement. Irreversible liver damage resulting from statins is extremely rare, with a liver failure rate of 1 case per 1 million person-years of use (Cohen, Anania, Chalasani, & National Lipid Association Statin Safety Task Force Liver Expert Panel, 2006).

However, even in the presence of statin intolerance symptoms, LDL-C reduction should be maximized and prioritized in patients with FH (Wiegman et al., 2015). Women should interrupt or be cautioned about continuing statin treatment during pregnancy and breastfeeding, owing to concerns over teratogenicity (Amundsen et al., 2006).

3.4.4.2. Non-statin drugs

In most patients with FH, statins alone are insufficient to normalize LDL-C levels, and ezetimibe and PCSK9 inhibitors should be added to control LDL-C levels more effectively. In some cases, the addition of ezetimibe to a statin is the preferred approach in the treatment of patients with FH (Watts et al., 2014). Some patients may require three or more medications to lower LDL-C adequately.

Fibrates are a class of amphipathic carboxylic acids. They are used for a range of metabolic disorders, mostly for triglyceride lowering but may have some LDL-C lowering effect. Ezetimibe, niacin, and bile acid sequestrants are also treatment options for drug intensification or for those intolerant of a statin. This should also be considered in FH patients who are not at very high risk when LDL-C does not decrease by 50% with statin monotherapy. It is important to note that doubling the dose of statin only achieves an additional LDL reduction by 6 -7% (Jones et al., 2003). Other options for those intolerant of statins include every other day statin therapy or lowering the dose while adding other treatment medications.

Ezetimibe is localized at the brush border of the small intestine and inhibits the absorption of cholesterol. It reduces LDL-C by about 15 to 20% when used alone and provides 20% percent additional reduction in combination with statins (Gagné, Gaudet, Bruckert, & Ezetimibe Study Group, 2002).

Bile acid sequestrants inhibit the enterohepatic reuptake and increase fecal loss of bile salts. They decrease LDL-C by preventing the reabsorption of bile acids in the terminal ileum. Because they are not absorbed systemically, they are considered safer to use than other cholesterol-lowering medications (Insull, 2006). Like ezetimibe, the effect on LDL-C reduction can be additive with statins and even ezetimibe (Huijgen et al., 2010). The need for suspensions or large numbers of pills, gastrointestinal side effects, and multiple drug-drug interactions limits patient adherence and use. Colesevelam, as compared to other bile acid sequestrants, has fewer gastrointestinal side effects and drug-drug interactions. Colesevelam is also approved for treatment of diabetes and may help patients achieve both glycemic and lipid goals (Zieve, Kalin, Schwartz, Jones, & Bailey, 2007).

Niacin, a water-soluble B vitamin, lowers LDL-C and raises HDL. It comes in crystalline and extended release forms. Due to concerns for liver toxicity, most non-prescription sustained release forms are not recommended (Ito et al., 2011).

PCSK9 inhibitors deliver a dose-dependent LDL cholesterol reduction of 50–65% in patients with FH and enable most patients to reach LDL cholesterol levels and prevention targets comparable to those of the general population (Qian et al., 2017). Data shows that PCSK9 inhibitors to statin treatment further reduces CVD events in high-risk patients with persistent inadequately controlled LDL cholesterol levels (Sabatine et al., 2017).

Lomitapide is an oral inhibitor of MTP, which is required for the assembly and secretion of lipoproteins that contain apoB, in conjunction with mipomersen which is an antisense oligonucleotide that binds to APOB mRNA and inhibits its translation, thereby reducing apoB synthesis and secretion are alternatives for patients who remain refractory to standard pharmacological treatments, especially patients with homozygous FH (Santos et al., 2016). In fact, lomitapide and mipomersen are only formally approved by the FDA for patients with homozygous FH, owing to their high cost and high rates of adverse effects, including hepatosteatosis (fatty liver disease) (Ginsberg et al., 2016).

3.4.4.3. Non pharmacological treatments

LDL apheresis is an important treatment modality for homozygous FH patients and for heterozygous patients who have not met treatment goals despite optimal tolerated medical therapy (Raal et al., 2011). It is an extracorporeal treatment that uses various methods to remove LDL from the circulation. LDL apheresis is currently FDA approved and has been shown in clinical trials to prevent and slow the progression of CHD (G. R. Thompson et al., 2010).

LDL apheresis is recommended for the following patients:

1. LDL goal reduction has not been achieved despite diet and maximum drug therapy (after 6 months).
2. Adequate drug therapy is not tolerated or contraindicated.
3. Functional homozygous FH patients with LDL-C \geq 300mg/dL (or non-HDL cholesterol \geq 330 mg/dL).
4. Functional heterozygous FH patients with LDL-C \geq 300 mg/dL (or non-HDL \geq 330 mg/dL).
5. Functional heterozygous FH patients with LDL-C \geq 200 mg/dL (or non – HDL cholesterol \geq 230 mg/dL) or high lipoprotein (a) \geq 50 mg/dL.
6. Functional heterozygotes with LDL cholesterol \geq 160 mg/dL (or non-HDL cholesterol \geq 190 mg/dL) and established CVD, other cardiovascular disease or diabetes.

Apheresis is generally done every 1 to 2 weeks with each session taking about 3 hours and removing greater than 60% of Apo-B containing lipoproteins (G. R. Thompson et al., 2010). The LDL reduction with LDL apheresis is temporary and associated with a rebound elevation in lipid levels after the procedure. The efficacy of LDL apheresis can be enhanced by the addition of statin therapy. LDL apheresis treatment in homozygous FH patients has improved their life expectancy to over 50 years. Cost and limited availability decrease widespread use of LDL apheresis.

For patients who do not achieve lipid goal reduction by the above modalities, other potential treatment options include partial ileal bypass and liver transplantation. Liver transplantation produces a significant lowering of LDL-C by providing normal LDL receptors. Nowadays, liver transplantation is used primarily in children with homozygous FH when apheresis is not an option or with concurrent heart transplantation (Palacio, Harring, Nguyen, Goss, & O'Mahony, 2011). Its use, however, is limited due to risk of transplant surgery and the limited number of donor livers. Partial ileal bypass is rarely used and works by interrupting enterohepatic bile acid circulation.

3.4.4.4. New approaches

Because interfering with PCSK9 function effectively reduces LDL cholesterol levels and CVD events, PCSK9 has become a target for lipid-lowering therapy using antibody-based drugs. Other approaches, such as gene silencing by RNA interference and antisense oligonucleotides, have been developed to interfere with PCSK9 production (Turner & Stein, 2015). The compound inclisiran, a long-acting small interfering RNA inhibitor of PCSK9 synthesis, was studied in 501 patients in a phase II study (Kausik K. Ray et al., 2017). Dose-dependent reductions in LDL cholesterol were observed at 6 months: reductions of 28–42% after a single dose of inclisiran and 36–53% after two doses. The two-dose inclisiran regimen reduced LDL cholesterol in 48% of the patients, and LDL cholesterol remained reduced compared with its baseline level after 8 months. Adverse events occurred slightly more often with inclisiran than with placebo (11% and 8%, respectively) (Kausik K. Ray et al., 2017). Antisense oligonucleotides are currently under development for other molecular targets, including for the inhibition of angiopoietin-related protein 3 (ANGPTL3, whose main function seems to be the regulation of lipoprotein lipase), MTTP and Lipoprotein (a) particles (Cupido et al., 2017).

Adnectins are fusion proteins derived from the tenth type III domain of human fibronectin and can bind to target proteins with high affinity and specificity. BMS962476, a PCSK9-targeted adnectin conjugated to polyethylene glycol, reduced LDL cholesterol by 48% and PCSK9 levels by >90% after subcutaneous injection in a phase I study (Turner & Stein, 2015). Additionally, in preclinical studies, peptide-based anti-PCSK9 vaccines showed a reduction of LDL cholesterol up to 55% for 1 year. In mouse models, these vaccines induced the generation of PCSK9 - specific antibodies that efficiently blocked murine PCSK9, upregulated murine LDL-R expression and recognized human PCSK9 (Galabova et al., 2014).

Numerous other LDL cholesterol-lowering agents are at various stages of development and might be available in the future for the management of at least some patients with heterozygous or homozygous FH, including the mentioned lomitapide and mipomersen as well as evinacumab (an antibody against ANGPTL3), bempedoic acid (an inhibitor of ATP citrate synthase, which is involved in the synthesis of acetyl-CoA and cholesterol) and gemcabene (which increases VLDL clearance) (Gryn & Hegele, 2016). Addition of daily oral anacetrapib, a CETP inhibitor, to baseline statin therapy over 4 years reduced well-controlled LDL cholesterol by 17% and CVD events by ~9% (REVEAL Collaborative Group et al., 2017). Whether these results are applicable to patients with FH is uncertain, but CETP inhibitors now represent a third category of non-statin agents (in addition to ezetimibe and PCSK9 inhibitors) that, when

added to a statin regimen, further reduce LDL cholesterol and CVD events in secondary prevention in the general population.

The ultimate cure for FH could be corrective gene therapy or gene replacement therapy, and although such potentially definitive treatments remain elusive, there are several promising investigational agents. An adeno-associated virus (AAV) vector encoding LDL-R cDNA induced a significant reduction of hypercholesterolemia in a humanized mouse model, which was *Ldlr-null* with transgenic expression of APOBEC, which encodes the catalytic component of the ApoB mRNA-editing enzyme complex. After injection of a low dose of the AAV vector containing LDL-R, the animals expressed human LDL-R and ApoB (Somanathan et al., 2014). Similarly, transfection with a vector expressing a gain of function LDL-R variant that is resistant to degradation by human PCSK9 and IDOL further reduced LDL cholesterol levels in mice (Kassim et al., 2013). If even one of these promising leads can be translated into a safe, effective and durable treatment, it would be transformational, particularly for patients with homozygous FH.

4. Objectives

We have mentioned what is cholesterol, how it is regulated and what are consequences of its accumulation by a pathological disorder as FH. However, if we look at the literature there are plenty studies about FH at organism level, but if we look closer and wonder “What about the cell?” There is almost no bibliography. Does the lack of cholesterol absorption affect the cell in any way? Theoretically the answer is no because cells can synthesize its own cholesterol as it is explained above. However, in practice, there are only a few papers which give some hints about what is going on in the cell of FH patients. As Descartes said “Divide each difficulty into as many parts as is feasible and necessary to resolve it”, maybe with a molecular point of view of FH we could not only learn more about FH but find new possible treatments far from the actual trends.

The initial objective of this thesis is to verify several molecular parameters of the FH related to the mutation in the LDL-R in patient fibroblasts in order to find new possible treatments. These will include levels, metabolism and synthesis of cholesterol, mitochondrial function, inflammation, autophagy and apoptosis. Due to the high relevance of this pathology in the large vessels, we will create an endothelium cell model by silencing the LDL-R and try to mimic the phenotype in this cell type. In this way, it can be corroborated and if the disease affects fibroblasts and endothelial cells in the same way. Because of the correlation between cholesterol and CoQ₁₀ biosynthesis, CoQ₁₀ pathways and regulation will be also studied.

The usage of human fibroblasts as cellular model in FH was previously corroborated by the experts of the cholesterol metabolism research Brown and Goldstein. In fact, they were given the Medicine Nobel Prize in 1985 using this model (Brown & Goldstein, 1996).

Once the necessary markers to complete a molecular analysis of the pathology have been obtained, an adequate treatment that reverses the symptoms and produces a beneficial effect on the cells will be applied.

The distribution of the study will be the following:

1. Controls and four FH patient fibroblast characterization:
 - a. General lipids and cholesterol accumulation observation by fluorescence (Filipin), Oil Red O staining and electron microscopy.
 - b. LDL-R levels by immunofluorescence and immunoblotting.
 - c. LDL uptake by fluorescent LDL.
 - d. Cholesterol and CoQ₁₀ biosynthesis by radioactive mevalonate assay.
 - e. Expression levels of biosynthesis and regulation related proteins by immunoblotting and qPCR.
 - f. Mitochondrial characterization:
 1. Reactive oxygen species production by flow cytometry.
 2. Mitochondrial membrane potential ($\Delta\Psi_m$) by flow cytometry.
 3. ATP production and bioenergetic profile by SeaHorse assay.
 4. Mitophagy activation by immunofluorescence and immunoblotting.
 5. Mitochondrial mass by flow cytometry, immunoblotting and spectrometry.
 - g. Autophagic flux by immunofluorescence and immunoblotting.
 - h. Cholesterol and CoQ₁₀ levels by HPLC.
 - i. *De novo* cholesterol and CoQ₁₀ biosynthesis by TLC chromatography.
 - j. Inflammation factors by immunoblotting and ELISA.
2. Generate and characterize an endothelial cell model:
 - a. Generate an endothelial FH cell model by LDL-R lentiviral silencing.
 - b. Characterizing the endothelial cell model:
 1. LDL uptake by fluorescent LDL.
 2. Expression levels of biosynthesis and regulation related proteins by immunoblotting.
 3. Cholesterol accumulation by fluorescence (Filipin).
 4. Membrane potential by flow cytometry.
3. Develop an adequate treatment for FH based on affected cells parameters and establish its effects on patient cells by the same methods of point "1. Controls and four FH patient fibroblast characterization."

5. Material and Methods

5.1. Reagents

Monoclonal anti-actin antibody, anti-VDAC1/Porin and anti-BECLIN1, rotenone, antimycin A and oligomycin were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against complex III (core 1 subunit), complex I (30 kDa subunit) and Complex IV (Cox II subunit), SREBP-2, RCAS (Golgi marker), polyclonal antibodies against INSIG2, NRLP3 and SCAP, MitoSox Red, CMH₂-DCFDA, 10-N-nonyl acridine orange (NAO), LysoTracker, tetramethylrhodamine methyl ester (TMRM), Fluorescent LDL Conjugates (DiI LDL) and Hoechst 33342 were obtained from Thermo Fisher Scientific/ Invitrogen/Molecular Probes (Waltham, MA). Anti-cytochrome c and anti-caspase 3 antibodies were obtained from BD Biosciences Pharmingen (San Jose, CA). Anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) monoclonal antibody (clone 6 C5) was from Calbiochem-Merck Chemicals Ltd. (Nottingham, UK). Anti-hATG12-ATG5 was obtained from Biosensis (South Australia, Australia). Anti-MAP-LC3 (N-20), anti-catalase (H-300), anti-PDI (H-160), anti-Golgi marker (AE-6), anti-cathepsin B, HMGCR, anti LDL-R, anti-caspase 1, anti-AMPK, anti-AMPK-p, anti-PPAR, anti-SREBP-1, anti-squalene synthase, anti-lanosterol synthase, anti-COQ1, anti-COQ2, anti-COQ7, anti-PCSK9, anti-ABCA1, anti-INSIG1, LDL-R shRNA and scramble shRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitors were obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma-Aldrich. Gap, PA, USA). 2-[¹⁴C]-(R, S)-mevalonate (55 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK).

All PBS (Phosphate Buffer Saline) used were 1x and pH 7.4, diluted from PBS 10x obtained from Intron Biotechnology.

5.2. Ethical statements

Approval of the ethical committee of the Hospital Universitario Virgen Macarena y Virgen de Rocío de Sevilla (Spain) was obtained, according to the principles of the Declaration of Helsinki and all the International Conferences on Harmonization and Good Clinical Practice Guidelines.

5.3. Fibroblasts cultures

Cultured fibroblasts were derived from a skin biopsy of patients (P1, P2, P3 and P4) with FH harboring heterozygous null mutations at the LDL-R gene:

- ➔ P1: carries the mutation c.1197_1205delCTACCTCTT
- ➔ P2 and P3: non-related patients with the same mutation, a deletion from exon 9 to exon 12, c.1187-?_1845+? del.
- ➔ P4: carries the mutation c.12G>A, p. (Trp4*).

Control fibroblasts were human skin primary fibroblasts from healthy volunteers. Samples from patients and controls were obtained according to the Helsinki Declarations of 1964, as revised in 2001. Fibroblasts from FH patients and controls were cultured at 37°C in DMEM (Dulbecco's Modified Eagle Medium) containing 4.5 g·L⁻¹ glucose, L-glutamine, and pyruvate supplemented with 1% antibiotic solution and 20% Fetal Bovine Serum (FBS).

5.4. LDL-R silencing cells

Endothelial cells (EA.hy926) were a generous donation from Carmelo Bernabeu Quirante (Centro Investigaciones Biológicas, CIB, Madrid, Spain). Cells were seeded in two 12-wells plates, silenced control and silenced LDL-R were grown in DMEM medium with 4,5 g·L⁻¹ glucose, 10% fetal bovine serum and 1% antibiotics (Optimal medium). After reaching a confluence of 50%, cells were washed once with PBS and replaced with optimal medium plus 10 µg/ml of Polybrene (Santa Cruz Biotech). shRNA Lentiviral particles (Santa Cruz Biotech) were added to the culture (shControl/shLDLR) and incubated overnight. Cell were washed with PBS once and replaced with optimal medium and incubated overnight. Every well were split in 3 different T25 Flasks and continue incubating for 48 hours in optimal medium. To select transfected and stable clones, the medium was supplemented with 0.5 µg/ml puromycin (Santa Cruz Biotechnology). Puromycin and medium was refreshed every week.

5.5. LDL uptake

The fluorescent-labeled LDL uptake assay was performed according to manufacturer's instructions (Thermo Fisher Scientific/Invitrogen/Molecular Probes). Cultured fibroblasts were incubated 2 hours at 37°C with culture medium mixed with human fluorescent LDL complexes at 12 µg/mL. After incubation, cells were rinsed twice with culture medium and visualized *in vivo* using an upright fluorescence microscope (Leica DMRE, Leica Microsystems GmbH, Wetzlar, Germany).

5.6. Oil red O staining

Oil red-O staining was performed as previously described (MCVEAN, PATRICK, & WITCHETT, 1965). Fibroblasts were grown on 1 mm width glass coverslips for 72 h in culture medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5' at room temperature, and permeabilized with 0.1% saponin for 5'. A 6:10 dilution was made from a 5% Oil red solution prepared in 2-propanol and added to cells for 60' at room temperature. The staining solution was removed and cells were washed with PBS three times before viewing by optical microscopy.

5.7. Filipin staining

Filipin staining was performed as previously described (Behnke, Trandum-Jensen, & van Deurs, 1984). Fibroblasts were grown on 1 mm width glass coverslips for 72 h in normal growth medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5' at room temperature and rinsed again. Cells were incubated for 10' at room temperature with 1.5 mg glycine/ml PBS to quench the paraformaldehyde. A working solution of 0.05 mg/mL Filipin (Sigma-Aldrich) in PBS was prepared and added to fibroblasts for 2 hours at room temperature. Cells were rinsed twice with PBS and visualized by fluorescence microscopy using a UV filter set. Filipin staining was quantified by measuring fluorescence intensity using the ImageJ software (NIH, USA).

5.8. Measurements of cholesterol and CoQ₁₀ levels

Cholesterol and CoQ₁₀ levels in cultured fibroblasts were measured using a method previously described by our group (Rodríguez-Hernández et al., 2009; Tang, Miles, DeGrauw, Hershey, & Pesce, 2001). Fibroblast pellets were lysed with 500µL PBS 1x and mixed vigorously. Then, 20 µL of 30 µM Coenzyme Q₉ was added as internal standard. 500µL SDS 2% and 2mL of ethanol:isopropanol (95:5) were added to each sample and mixed. To extract lipids, 4 ml of hexane were added and vortexed. Samples were centrifuged at 1000×g for 5' at 4 °C and the upper phase was recovered. This step was repeated twice and 12mL were dried via rotary evaporation. Lipid extracts were vortexed with 900µL of ethanol (HPLC quality) and dried in a speed-vac. The lipid pellet was suspended and homogenized in 100µL of ethanol prior to HPLC injection. Lipid components were separated by a SHIMADZU UFLC HPLC system equipped with a reversed-phase Shim-pack XR-ODS C-8 column in a column oven set to 40 °C, with a flow rate of 0.5 ml/minute and a mobile phase containing 80:20 methanol/2-propanol. Coenzyme Q₉ (internal standard), CoQ₁₀ and cholesterol levels were analyzed with a SPD-20A prominence UV-VIS detector at 200 and 245nm, respectively.

5.9. *De novo* synthesis of cholesterol

The rates of synthesis of cholesterol and CoQ₁₀ were measured following a protocol previously described (Nambudiri, Ranganathan, & Rudney, 1980). Fibroblast were incubated with 2-[¹⁴C]-(R, S) - mevalonolactone (55 mCi/mmol, Amersham, Buckinghamshire, UK) at a concentration of 10,000 dpm/mL and 20 µM unlabeled mevalonolactone (Sigma-Aldrich) in the media for 48h at 37°C and 5% CO₂. Lipid extraction was developed as indicated previously. The resulting lipid extracts were complemented with 5 µg of cholesterol and CoQ₁₀ and fractionated by thin layer chromatography (TLC) on 20x20 cm silica gel plates Si60 (Merck Millipore, Darmstadt, Germany) activated for 1h at 100°C. The mobile phase was hexane/ethyl ether 80:20. The bands corresponding to cholesterol and CoQ₁₀ were located by exposing the plate to iodine vapor, and the bands were scrapped off from the plates and the silica gel transferred to scintillation vials. The radioactivity incorporated in the bands was measured by liquid scintillation in a Beckman Coulter SL6500 counter and the rates of synthesis calculated attending to the specific radioactivity of the precursor.

5.10. Immunofluorescence microscopy

Fibroblasts were grown on 1 mm width glass coverslips for 72 h in normal growth medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5' at room temperature, and permeabilized with 0.1% saponin for 5'. Glass coverslips were incubated at 37°C with primary antibodies diluted 1:100 in PBT for an hour and rinsed twice with PBS. The secondary antibody, diluted 1:100 in PBS, was incubated for 45' at 37°C. The coverslips were then rinsed twice with PBS, incubated for 4' with PBS containing Hoechst 33342 dilution 1:1000 and washed with PBS. The coverslips were mounted onto microscope slides using Vectashield Mounting Medium and analyzed using an upright fluorescence microscope (Leica DMRE, Leica Microsystems GmbH, Wetzlar, Germany). Images were taken using a DeltaVision system (Applied Precision; Issaquah, WA, USA) with an Olympus IX-71 microscope using a 100× objective.

5.11. Immunoblotting

Western blotting was performed using standard methods. After transferring the proteins, the membranes were incubated with primary antibodies at 1:1000, overnight, rinsed twice and incubated again with the corresponding secondary antibody. Multiple blots were run and several proteins of interest were serially detected. Every membrane was checked for protein loading using Ponceau staining and actin expression levels. Stripping was not used. As possible each membrane was re-probed with different antibodies if the molecular weight of the protein did not interfere with the previous one. If the proteins were sufficiently separated from one another during gel electrophoresis, then membranes were cut and each respective piece was used to detect the appropriate protein.

5.12. ATP levels

An ATP determination kit (Invitrogen-Molecular Probes) was used to measure ATP levels by a bioluminescence assay.

5.13. Mitochondrial membrane potential

FH fibroblasts were grown on multiwell plates for 24 to 48 hours, incubated with 100 nM TMRM for 30' and examined by flow cytometry. Treatment of control cells with 20 μ M FCCP for 20' was used as positive control of $\Delta\Psi_m$ depolarization.

5.14. Mitochondrial mass

Mitochondrial mass was determined by flow cytometry after cell staining with 10 μ M 10-N-nonyl acridine orange (NAO) for 10' at 37°C in the dark. Alternatively, mitochondrial mass was assessed measuring citrate synthase activity (Rustin et al., 1994) .

5.15. Mitochondrial reactive oxygen species

Mitochondrial ROS generation in fibroblasts was assessed by MitoSOX, a red mitochondrial superoxide indicator. Once in the mitochondria, MitoSOX red reagent is oxidized by superoxide and exhibits red fluorescence. Approximately 1×10^6 cells were incubated with 1 μ M MitoSox for 30' at 37°C, washed twice with PBS, resuspended in 500 μ l of PBS, and analyzed by flow cytometry (excitation at 510 nm and fluorescence detection at 580 nm). Specificity of MitoSOX for superoxide has been shown by the manufacturer, and its mitochondrial localization was tested by co-staining with MitoTracker Green (data not shown). ROS levels were expressed relative to the mitochondrial mass (ROS signal/NAO signal).

5.16. Mitochondrial respiratory chain activity

Activities of NADH cytochrome c reductase (complexes I+III) and citrate synthase were determined spectrophotometrically in sonicated and permeabilized fibroblasts using previously described methods (Rustin et al., 1994). Results are expressed as units/citrate synthase (mean \pm SD). Protein content was determined by the Lowry procedure.

5.17. Real-time quantitative PCR

The fibroblast expression of ATG12, MAP-LC3, BECLIN1, COQ10 1, COQ10 2, SREBP-2, HMGCR and LSS (Lanosterol Synthase) genes in fibroblasts was analyzed by SYBR Green quantitative PCR using mRNA extracts and primers.

- ➔ Human BECLIN1 primers 5-GGATGGATGTGGAGAAAGGCAAG-3 (forward) and 5-TGAGGACACCCAAGCAAGACC-3 (reverse) amplify a sequence of 152 nt.
- ➔ Human ATG12 primers 5-ATTGCTGCTGGAGGGGAAGG-3 (forward) and 5-GGTTCGTGTTGCTCTACTGC-3 (reverse) amplify a sequence of 198 nt.
- ➔ Human MAP-LC3 primers 5-GCCTTCTTCCTGCTGGTGAAC-3 (forward) and 5-AGCCGTCCTCGTCTTTCTCC-3 (reverse) amplify a sequence of 91 nt.
- ➔ Human COQ1 primers 5-TTCAACAGCGACACCCACT-3 (forward) and 5-CTGCAATGGACTAGCTCTGC-3 (reverse) amplify a sequence of 179 nt.
- ➔ Human COQ2 primers 5-CGCAGAGCCGTTGACTTAAG-3 (forward) and 5-GCGTTACTTGATGGTCTGC-3 (reverse) amplify a sequence of 160 nt.
- ➔ Human SREBP-2 primers 5-GGAGTGGTGCTGAATGTTGG-3 (forward) and 5-TTTCTCCACCTCAGTTCCTCC-3 (reverse) amplify a sequence of 132 nt.
- ➔ Human HMGCR primers 5-CACAACAAGCTCCCATCACC-3 (forward) and 5-GGAAACTCATGAGCGTGGTG-3 (reverse) amplify a sequence of 127 nt.
- ➔ Human LSS primers 5-CCCAACACAGTTCCTTCAGC-3 (forward) and 5-TCTTCTGTCCAGCTCCCTTG-3 (reverse) amplify a sequence of 163 nt.
- ➔ Human GAPDH primers 5-TTGAGGTCAATGAAGGGGTC-3 (forward) and 5-GAAGGTGAAGGTCGGAGTCA-3 (reverse) amplify a sequence of 185 nt. GAPDH was used as control gene.

5.18. Electron microscopy

Electron microscopy was performed as described (Rodríguez-Hernández et al., 2009). Culture cells were fixed with 2% glutaraldehyde for 15' at RT and for 30' in 2% glutaraldehyde 0.1M NaCacodylate/HCl at pH 7.4. Fibroblasts were washed with 0.2 M NaCacodylate/ HCl, pH 7.4 and fixed again for 30' 1% OsO₄ 0.15 M NaCacodylate/HCl, pH 7.4. Samples were dehydrated with ethanol at 30, 50, 70 and 95% for 5j. Impregnation and inclusion steps were performed in Epon and polymerized for 48h at 60°C. An ultramicrotome RMC-MTX (Tucson, Arizona) was used to get 60-80 nm sections. Sections were contrasted with uranyl acetate and lead citrate. Philips CM-10 transmission electron microscope was used to capture images.

5.19. Lysosomal content

Fibroblasts were cultured in multiwell plates and incubated for 30' at 37°C with 100nM LisoTracker Red™ (Molecular Probes). After incubation, cells were washed and the red fluorescence was measured by flow cytometry.

5.20. Interleukins

IL-1 β and IL-18 levels in culture mediums were assayed by commercial ELISA kits (MyBioSource, Inc., CA, USA).

5.21. Bioenergetics

Mitochondrial respiratory function of control and FH fibroblasts were measured using mito-stress test assay by XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) according to manufacturer's instructions. Cells were seeded at a density of 3x10⁴ cells/well in XF24 cell culture plates in 100 μ L growth medium (DMEM medium containing 20% FBS) and placed in 37°C incubator with 5% CO₂. After 24 h incubation, growth medium from each well were removed, leaving 50 μ L of media. Then, cells were washed twice with 1,000 μ L of pre-warmed assay medium (XF base medium supplemented with 10 mM glucose, 1 mM glutamine and 1 mM sodium pyruvate; pH 7.4) and 450 μ L of assay medium (500 μ L final) was added. Cells were incubated in 37°C incubator without CO₂ for 1 h to allow pre-equilibrating with the assay medium. Mitochondrial functionality was evaluated by sequential injection of four

compounds that affect bioenergetics. The final concentrations of injections were: 1 μ M oligomycin, 2 μ M FCCP, 1 and 2.5 μ M rotenone/antimycin A. The best concentration of each inhibitor and uncoupler, as well as the optimal cells seeding density were determined in preliminary analyses. A minimum of five wells per treatment were utilized in any given experiment. This assay allowed for an estimation of basal respiration, maximal respiration and spare respiratory capacity.

5.22. Statistical analysis

We used non-parametric statistics that do not have any distributional assumption, given the low reliability of normality testing for small sample sizes used in this work (Le Boedec, 2016). To compare parameters between groups, variables were evaluated using Wilcoxon match-paired signed rank test, Friedman Test or 2-way ANOVA Test. All results are expressed as mean \pm SD of 3 independent experiments and a p-value<0.05 was considered as statistically significant.

6. Results

6.1. Cholesterol accumulation in fibroblasts derived from patients harboring LDL-R mutations

In order to determine whether there was any evidence of defective LDL-R function in fibroblasts derived from FH patients, an *in vitro* LDL uptake assay was performed. Fluorescent labeled LDL complexes were used to evaluate the incorporation of cholesterol mediated by LDL-R in control and patient fibroblasts. All patient fibroblasts showed, as expected, a decrease of $60\pm 10\%$ of LDL uptake compared to control fibroblasts (**Figure 8 A and B**). Decreased LDL uptake was associated with reduced expression levels of LDL-R in FH fibroblasts assessed by Western blotting (**Figure 8 C and D**) and immunofluorescence microscopy (**Figure 9**).

Given that FH fibroblasts showed decrease LDL-C uptake, we next examined intracellular lipids in control and patient fibroblasts by oil red staining, a lysochrome commonly used to stain neutral lipids including esterified cholesterol. A significant increase in oil red staining was found in patient fibroblasts (**Figure 8 E and F**). Intracellular lipid accumulation was confirmed by electron microscopy examination that showed the presence of lipid droplets inside patient fibroblasts (**Figure 8 G and H**). To characterize lipid accumulation, cells were also stained with Filipin, a highly fluorescent polyene macrolide antibiotic which binds specifically to unesterified cholesterol but not to esterified sterols. Filipin staining was notably increased in patient fibroblasts indicating increased cholesterol content in patient cells (**Figure 8 I and J**).

Mevalonate pathway is an important metabolic pathway which plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA and CoQ₁₀ (Buhaescu & Izzedine, 2007). It has been suggested that there is a relation between cholesterol and CoQ₁₀ levels due to the fact that both compounds are synthesized in the same pathway (Turunen, Olsson, & Dallner, 2004).

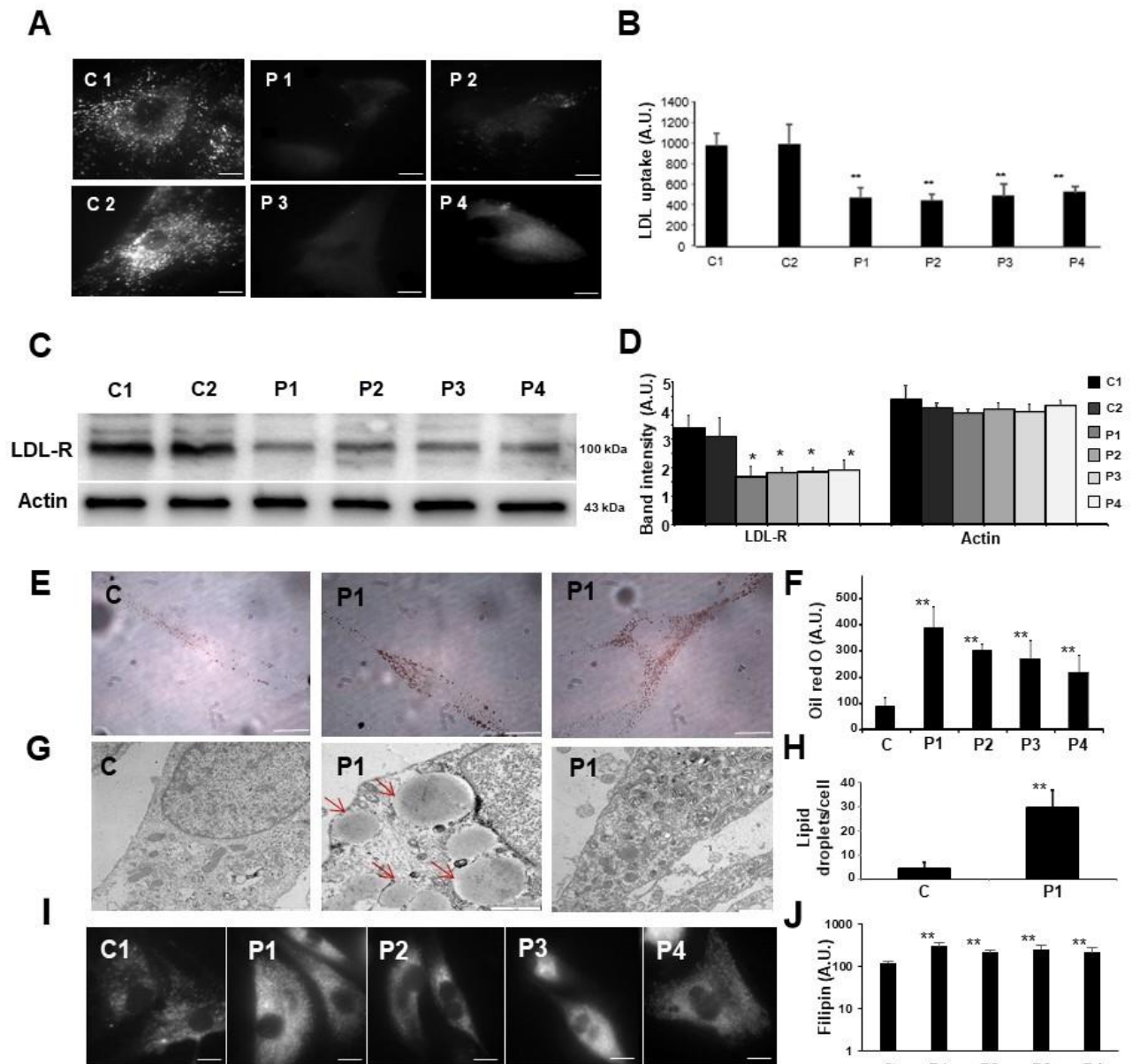


Figure 8. Impaired LDL uptake and cholesterol accumulation in FH fibroblasts. (A) Control and FH fibroblasts were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy. An impaired LDL-uptake was found in FH fibroblasts. Scale bar =15 μ m. (B) Quantification of LDL-uptake by Image-J software. (C) LDL-R expression. Expression levels of LDL-R in control and FH fibroblasts determined by Western blotting. Actin was used as a loading control. For control cells, data are a pool of 2 different control cell lines. (D) Quantification of LDL-R Western blotting by Image-J software. (E) Oil red staining in control and FH fibroblasts. Scale bar =15 μ m. (F) Image analysis of oil red staining. (G) Electron Microscopy in control and FH fibroblasts. Red arrows indicate lipid droplets. Scale bar =1 μ m. (H) Quantification of lipid droplets. (I) Filipin staining in control and FH fibroblasts. Cells were then examined under fluorescence microscopy. Scale bar =15 μ m. (J) Filipin quantification by Image J software. Data represent the mean \pm SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts. **p<0.01 between control and FH fibroblasts.

A.U., arbitrary units; FH, Familial Hypercholesterolemia; LDL, Low-Density Lipoprotein; LDLR, LDL receptor; SD, Standard Deviation

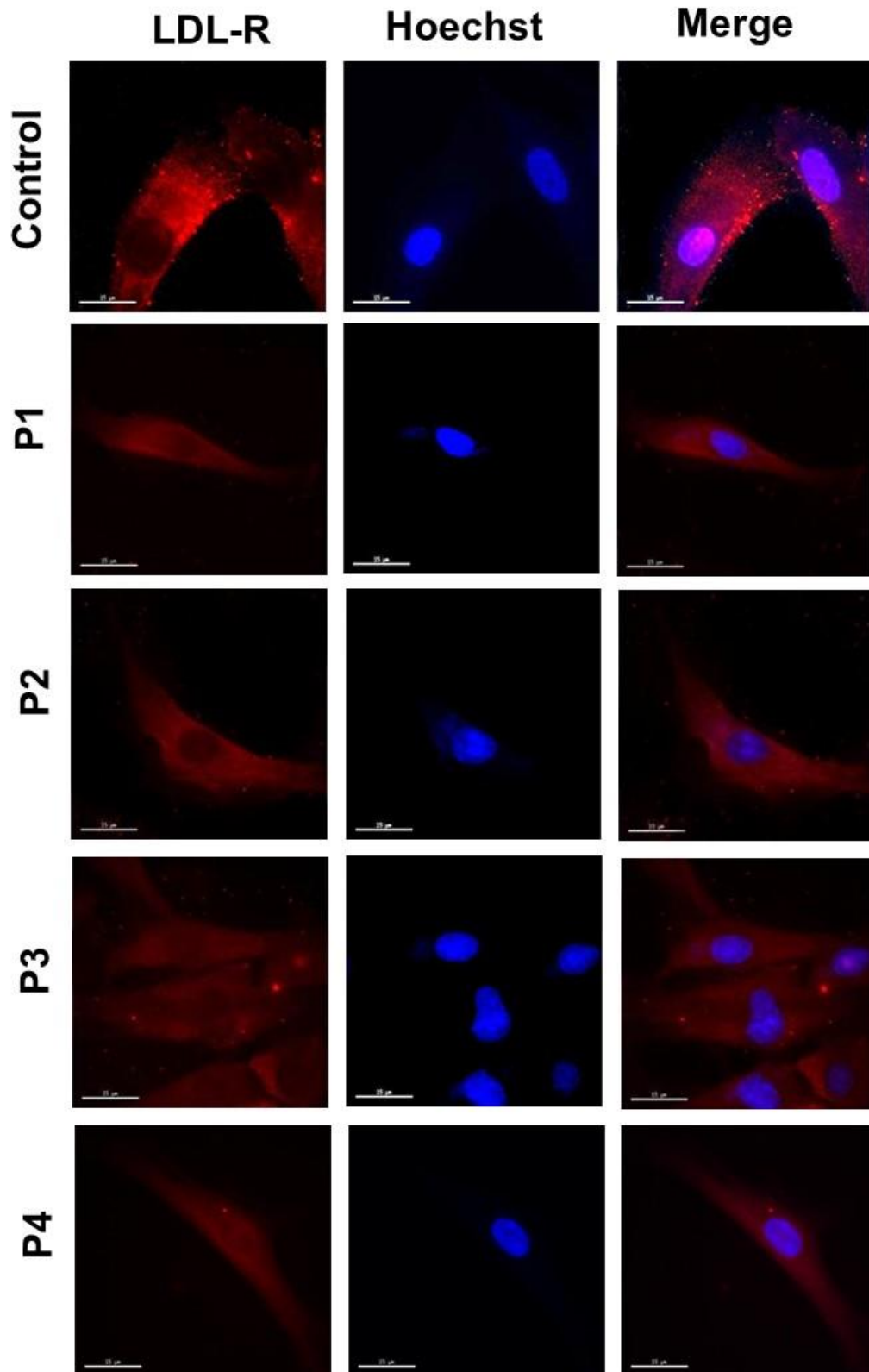


Figure 9. Immunofluorescence of LDL-R. Control and FH fibroblasts were fixed and immunostained with anti-LDL-R and examined by fluorescence microscopy. Nuclei were revealed by Hoechst staining. Scale bar=15 μ m.

LDL-R, Low Density Lipoprotein Receptor; FH, Familial Hypercholesterolemia

To explore mevalonate pathway in FH fibroblasts, we decided to examine *de novo* synthesis of two of the main final product of this pathway, cholesterol and CoQ₁₀. Radioactive labelled mevalonate was incorporated to cells and cholesterol and CoQ₁₀ levels were examined by thin layer chromatography (TLC). The radioactivity incorporated in cholesterol was significantly increased (**Figure 10 A**) while the radioactivity incorporated in CoQ₁₀ was significantly reduced in FH patients (**Figure 10 B**). These findings made us suspect that elevated cholesterol biosynthesis might have induced increased cholesterol levels and a secondary deficiency of CoQ₁₀. To asses this hypothesis, cholesterol and CoQ₁₀ levels were measured in control and FH fibroblasts by HPLC. Cholesterol levels were markedly increased (**Figure 10 C**) while CoQ₁₀ levels were significantly reduced (**Figure 10 D**) in patient fibroblasts.

To further examine the mechanism of dysregulated mevalonate pathway in FH, the expression levels of proteins involved in cholesterol and CoQ₁₀ biosynthesis regulation were analyzed by Western blotting. The mature form of transcription factor SREBP-2 and SREBP-1 which regulates the protein expression of key enzymes involved in cholesterol and lipid biosynthesis were increased in FH fibroblasts (**Figure 11 and Figure 12**). Accordingly, the expression levels of HMGCR, lanosterol synthase and squalene synthase were upregulated. Concomitantly, FH fibroblasts showed reduced expression of proteins involved in CoQ₁₀ biosynthesis (COQ1, COQ2 and COQ7) (**Figure 11, Figure 12 A and B**). Correspondingly, these alterations of protein expression levels were accompanied by increased expression of cholesterologenic transcripts and decreased expression of COQ genes (**Figure 12 C**).

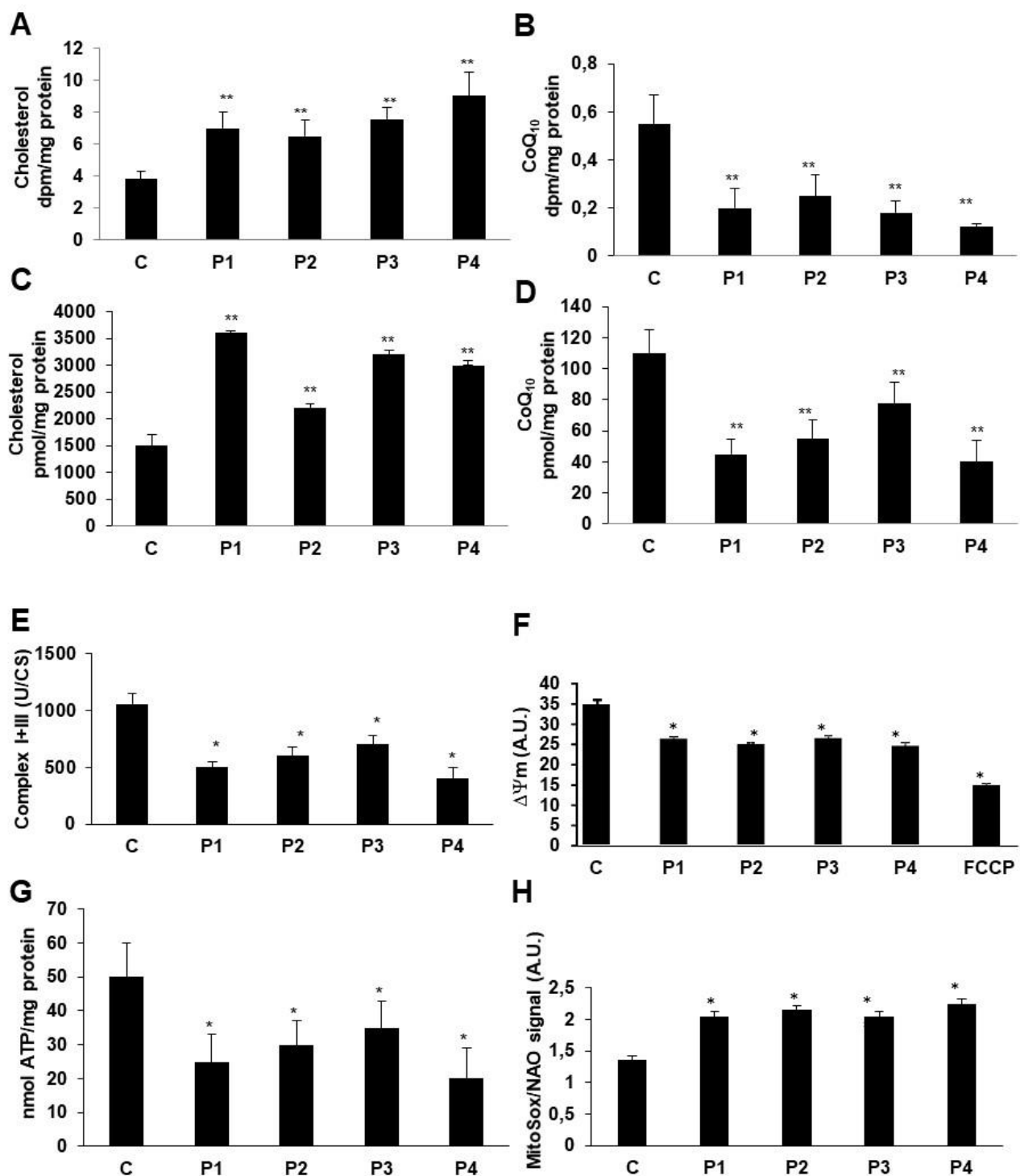


Figure 10. Dysregulated mevalonate pathway and mitochondrial dysfunction in FH fibroblasts. A and B: Cholesterol (A) and CoQ₁₀ (B) biosynthesis analysis in control and FH fibroblasts was performed incubating cells with radioactive mevalonate. For control cells, the data are the mean±SD for experiments conducted on 2 different control cell lines. (C) Cholesterol levels in control and FH fibroblasts. (D) CoQ₁₀ levels in control and FH fibroblasts. (E) Mitochondrial enzymatic activities of complex I+III in control and FH fibroblasts. (F) $\Delta\Psi_m$ was assessed by flow cytometry using TMRM. A clear decrease of $\Delta\Psi_m$ was observed in FH fibroblasts. Treatment of control cells with 20 μ M FCCP for 20' was used as positive control of $\Delta\Psi_m$ depolarization. (G) ATP levels in control and FH fibroblasts. A significant decrease of ATP levels was observed in FH fibroblasts. (H) Mitochondrial ROS levels in control and FH fibroblasts. Results are expressed as the ratio of MitoSOX signal to 10-N-nonyl acridine orange signal. For control cells, the data are the mean±SD for experiments conducted on 3 different control cell lines. Data represent the mean±SD of 3 separate experiments. * $p < 0.05$ between control and FH fibroblasts. ** $p < 0.01$ between control and FH fibroblasts. A.U., arbitrary units.

CoQ₁₀, Coenzyme Q₁₀; FH, Familial Hypercholesterolemia; $\Delta\Psi_m$, Mitochondrial membrane potential; TMRM, Tetramethylrhodamine; FCCP, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ATP, Adenosin triphosphate; ROS, Reactive Oxygen Species

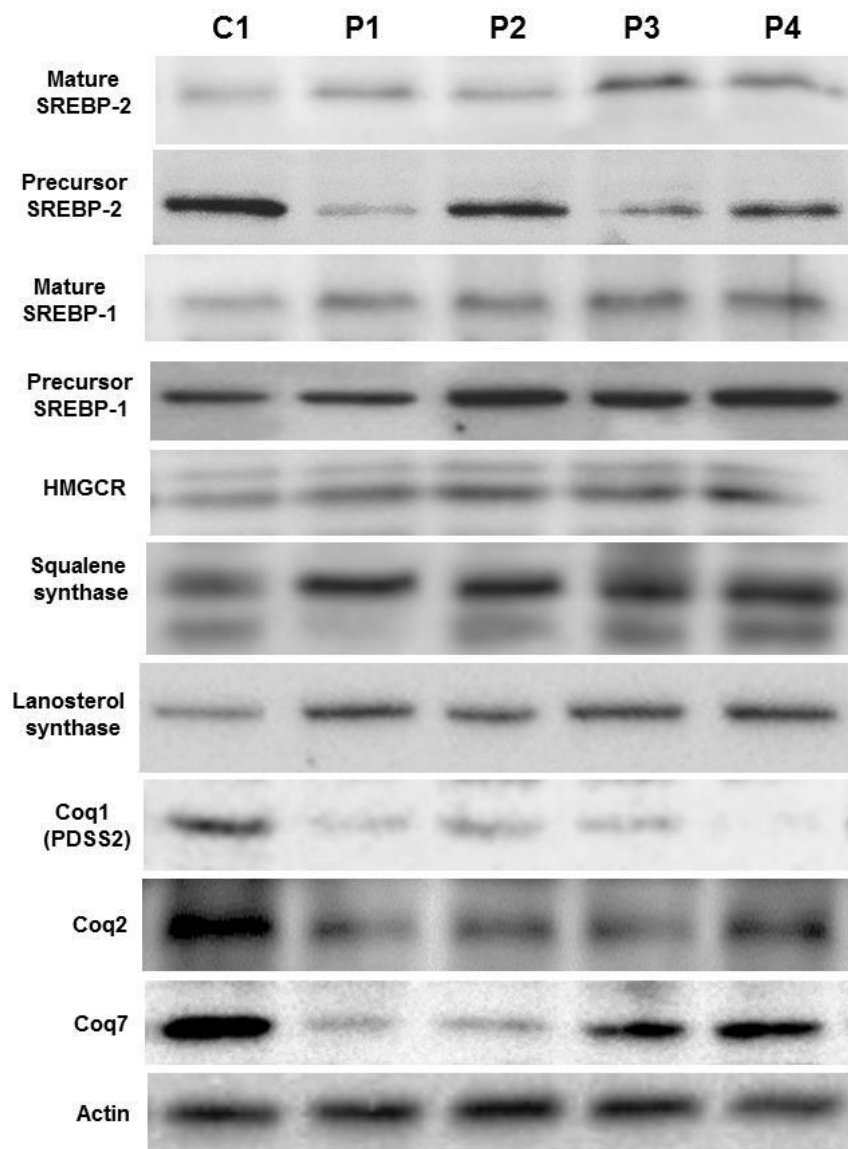


Figure 11. Expression levels of proteins involved in cholesterol. SREBP-2, HMGCR, lanosterol and squalene synthase and CoQ₁₀ (COQ1, COQ2 and COQ7) biosynthesis in control and FH fibroblasts were analyzed by Western blotting. Actin was used as a loading control.

SREBP-2, Sterol regulatory element-binding protein 2; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; CoQ₁₀, Coenzyme Q₁₀; FH, Familial Hypercholesterolemia

6.2. Mitochondrial dysfunction in FH fibroblasts

CoQ₁₀ is an essential component of the mitochondrial electron transport chain (ETC) by transferring reducing equivalents from complexes I and II to complex III (Crane, 2001). In order to find out if CoQ₁₀ deficiency also affects mitochondrial function in FH fibroblasts, we measured the activities of mitochondrial respiratory chain enzymes. Complex I+III activity was significantly reduced in patient compared to control fibroblasts (**Figure 10 E**). As reduced mitochondrial complexes activity may lead to mitochondrial dysfunction, we next examined $\Delta\Psi_m$ and mitochondrial ROS production. **Figure 10 F** shows that $\Delta\Psi_m$ was reduced in patient fibroblasts. To assess if cellular bioenergetics was also affected in patient fibroblasts, intracellular ATP levels were also measured. A decrease of $45\pm13\%$ was found in patient fibroblasts (**Figure 10 G**). In addition, mitochondrial ROS levels were significantly increased in FH fibroblasts (**Figure 10 H**).

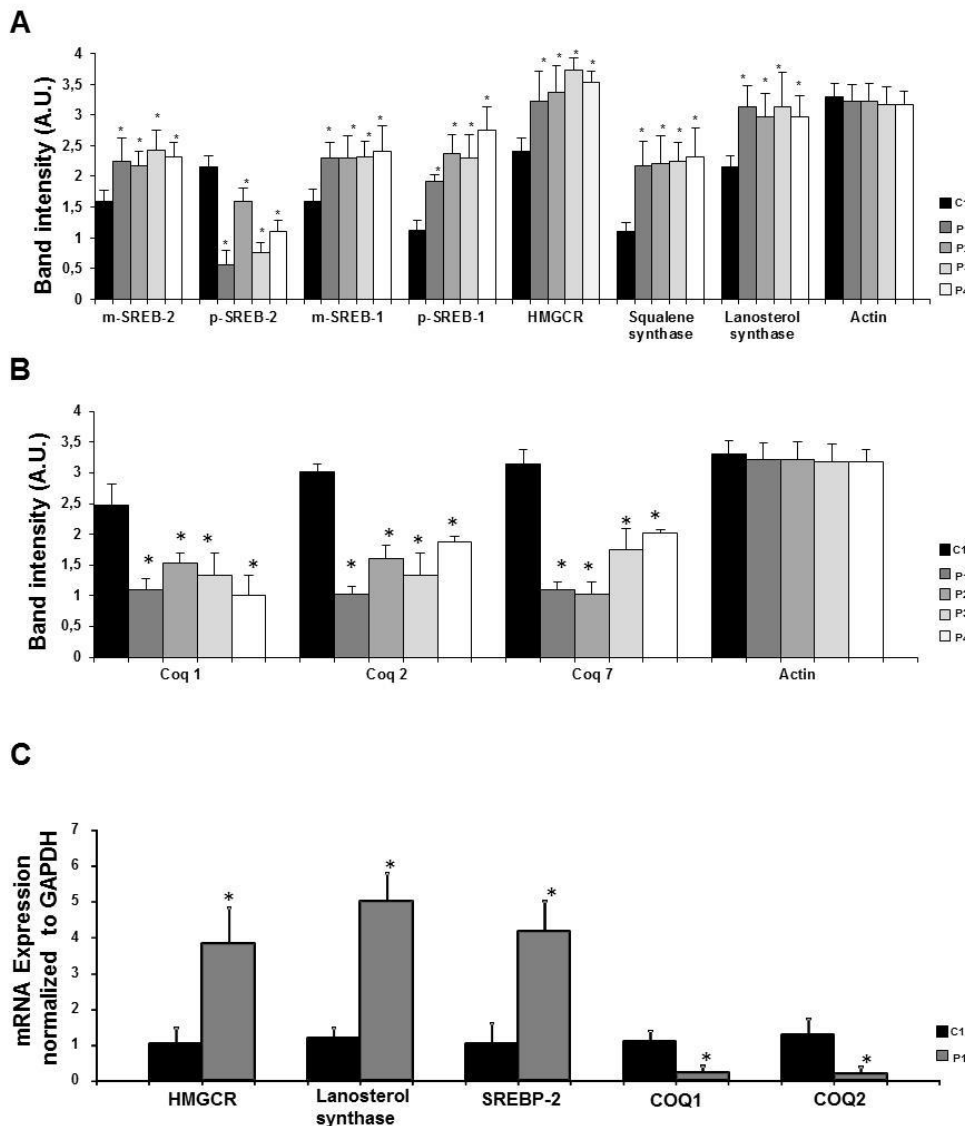


Figure 12. Cholesterogenic and coenzyme Q₁₀ genes expression. A and B densitometry of Western blotting of **Figure 11**. (C) Expression levels of cholesterogenic and CoQ₁₀ biosynthetic transcripts. Expression levels of HMGCR, lanosterol synthase, SREBP-2, COQ1 and COQ2 transcripts were performed by RT-PCR. Data represent the mean \pm SD of 3 separate experiments. *p<0.01 between control and FH fibroblasts.

A.U., arbitrary units; CoQ₁₀, Coenzyme Q₁₀; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; SREBP-2, Sterol regulatory element-binding protein 2; RT-PCR, Real Time Polymerase Chain Reaction; SD, Standard Deviation; FH, Familial Hypercholesterolemia

6.3. Selective degradation of mitochondria in FH fibroblasts

As mitochondria dysfunction is often associated with mitophagy activation as a mechanism for the elimination of damaged mitochondria (Cotán et al., 2016; De la Mata et al., 2012), we next evaluated the amount of acidic vacuoles and the expression levels of autophagy proteins. Acidic vacuoles were examined by LysoTracker red staining coupled to flow cytometry analysis. Higher lysosomal content was found in FH fibroblasts respect to control cells (**Figure 13 A**).

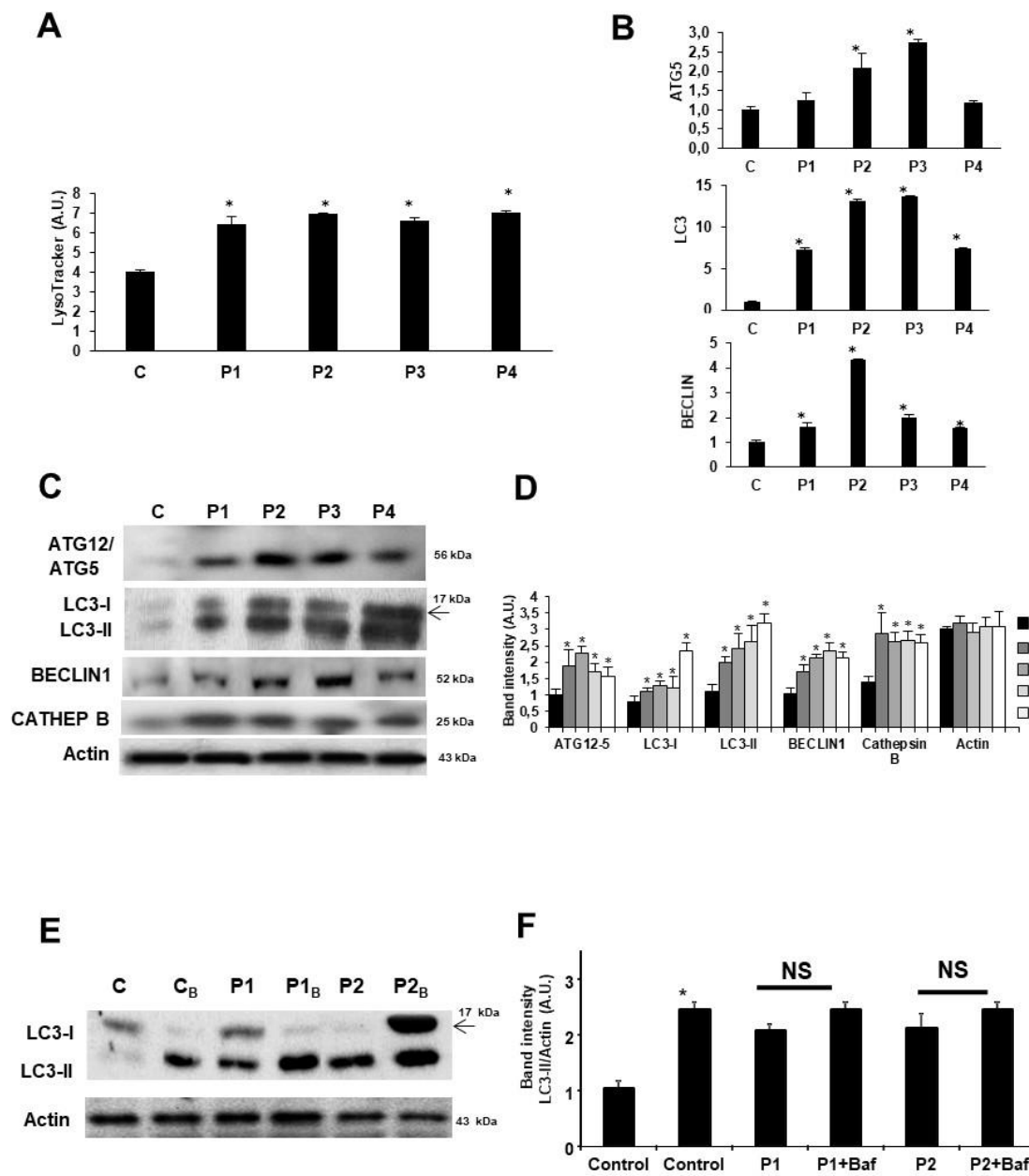


Figure 13. Increased expression of autophagic markers in FH fibroblasts.

(A) Quantification of acidic vacuoles in control and FH fibroblasts by LysoTracker staining and flow cytometry analysis. (B) Increased expression of autophagic transcripts. Expression levels of BECLIN1, LC3 and ATG5 were performed by RT-PCR. (C) Increased expression of autophagic proteins. The expression levels of LC3-I (upper band) and LC3-II (lower band), ATG12, BECLIN1 and cathepsin D were determined in the control and FH fibroblast cultures by Western blotting. The ATG12 band represents the ATG12-ATG5 conjugated form. Actin was used as a loading control. (D) Densitometric analysis of Western blottings. (E) Autophagy flux in FH fibroblasts. Determination of LC3-II levels in the presence and absence of bafilomycin A1 in control and FH fibroblasts. Control and FH fibroblasts were incubated with bafilomycin A1 (100 nM for 12 h). Total cellular extracts were analyzed by immunoblotting with antibodies against LC3. Actin was used as a loading control. (F) Densitometry of Western blotting was performed using the ImageJ software. For control cells, the data are the mean±SD for experiments conducted on 3 different control cell lines. Data represent the mean±SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts.

FH, Familial Hypercholesterolemia; LC3, Microtubule-associated proteins 1A/1B light chain 3B; ATG5, Autophagy Related 5; RT-PCR, Real Time Polymerase Chain Reaction; ATG12, Autophagy Related 12; A.U., Arbitrary Units; SD, Standard Deviation.

To assess that high lysosomal content was accompanied by activation of autophagic processes, mRNA levels and proteins connected to autophagy were examined. The expression of BECLIN1, LC3 and ATG5 genes determined by quantitative PCR displayed an increase in FH patients respect to control fibroblasts (**Figure 13 B**). The same pattern was found when protein levels of ATG5-12, LC3-I and LC3-II were examined by Western blot (**Figure 13 C and D**). Increased levels of cathepsin B (CATHEP B), a lysosomal protein, were also found increased in patient fibroblasts compared to control fibroblasts.

Next, autophagy flux was examined by treating control and FH fibroblast with bafilomycin A1, a vacuolar H⁺ ATPase inhibitor that prevents the fusion between autophagosomes and lysosomes (Yamamoto et al., 1998), and analyzing LC3-II expression levels by Western blotting. Control cells treated with bafilomycin showed increased LC3-II expression levels indicating that autophagy flux was normal (**Figure 13 E and F**). In contrast, the treatment with bafilomycin in mutant fibroblasts (P1 and P2) induced only a slight increase in LC3-II expression levels suggesting that autophagy flux was impaired in FH fibroblasts (**Figure 13 E and F**).

To assess the selective elimination of mitochondria in FH cells, protein expression levels of mitochondrial proteins were examined. Expression levels of mitochondrial proteins, VDAC/porine, Complex I (30 kDa subunit) and complex IV (Cox II subunit) were markedly reduced in FH fibroblasts (**Figure 14 A**). In contrast, protein expression levels of markers of other organelles such as endoplasmic reticulum (PDI), Golgi apparatus (RCAS) and peroxisomes (catalase) were not affected in patient fibroblasts, suggesting that only mitochondria were selectively eliminated in FH fibroblasts (**Figure 14 A and B**). Reduced mitochondrial mass was also confirmed by measuring citrate synthase activity, a well-known marker of mitochondria abundance. Citrate synthase activity was dramatically reduced in patient fibroblasts, indicating extensive mitochondrial loss and/or impaired mitochondrial biogenesis (**Figure 14 C**).

Mitophagy was also assessed by immunofluorescence microscopy by the examination of co-localization of the autophagosome marker LC3 and the mitochondrial marker cytochrome c. High co-localization between mitochondria and autophagosomes markers was found in 80% of analyzed cells in patient fibroblasts (**Figure 15 A and B**).

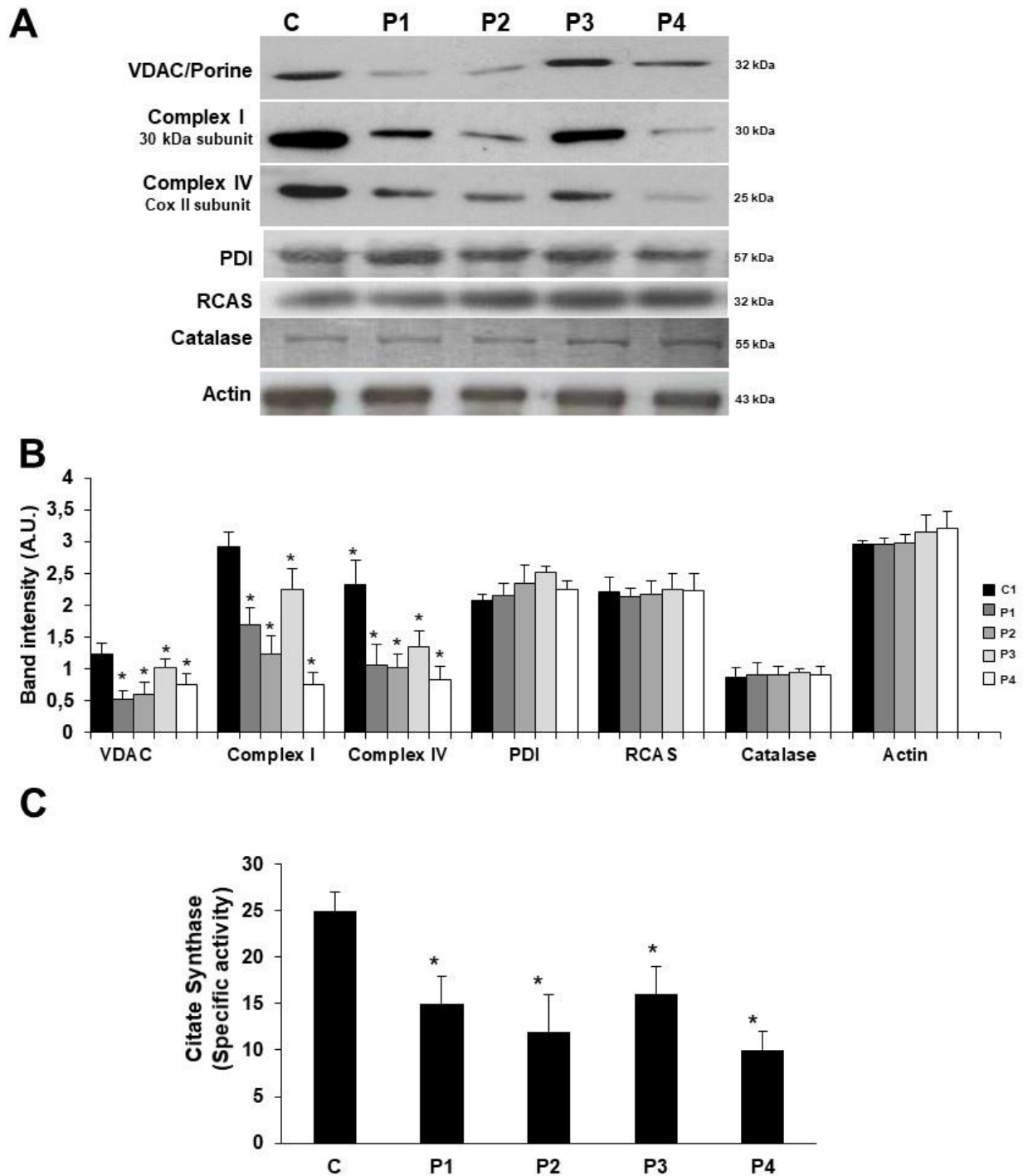


Figure 14. Mitophagy in FH fibroblasts. (A) Western blot analysis of mitochondrial (complex I, 30 kDa subunit; complex IV, COX II subunit; and VDAC/porin), Golgi (Golgi marker), endoplasmic reticulum (PDI), and peroxisome (catalase) proteins in control and FH fibroblasts. Actin was used as loading control. (B) Densitometry of Western blotting. (C) Mitochondrial mass was determined by determining citrate synthase activity. For control cells, the data are the mean \pm SD for experiments conducted on 3 different control cell lines. Data represent the mean \pm SD of 3 separate experiments. * $p < 0.05$ between control and FH fibroblasts.

A.U., arbitrary units; VDAC, Voltage-dependent anion channels; PDI, Protein disulfide isomerase; FH, Familial Hypercholesterolemia; SD, Standard Deviation; FH, Familial Hypercholesterolemia

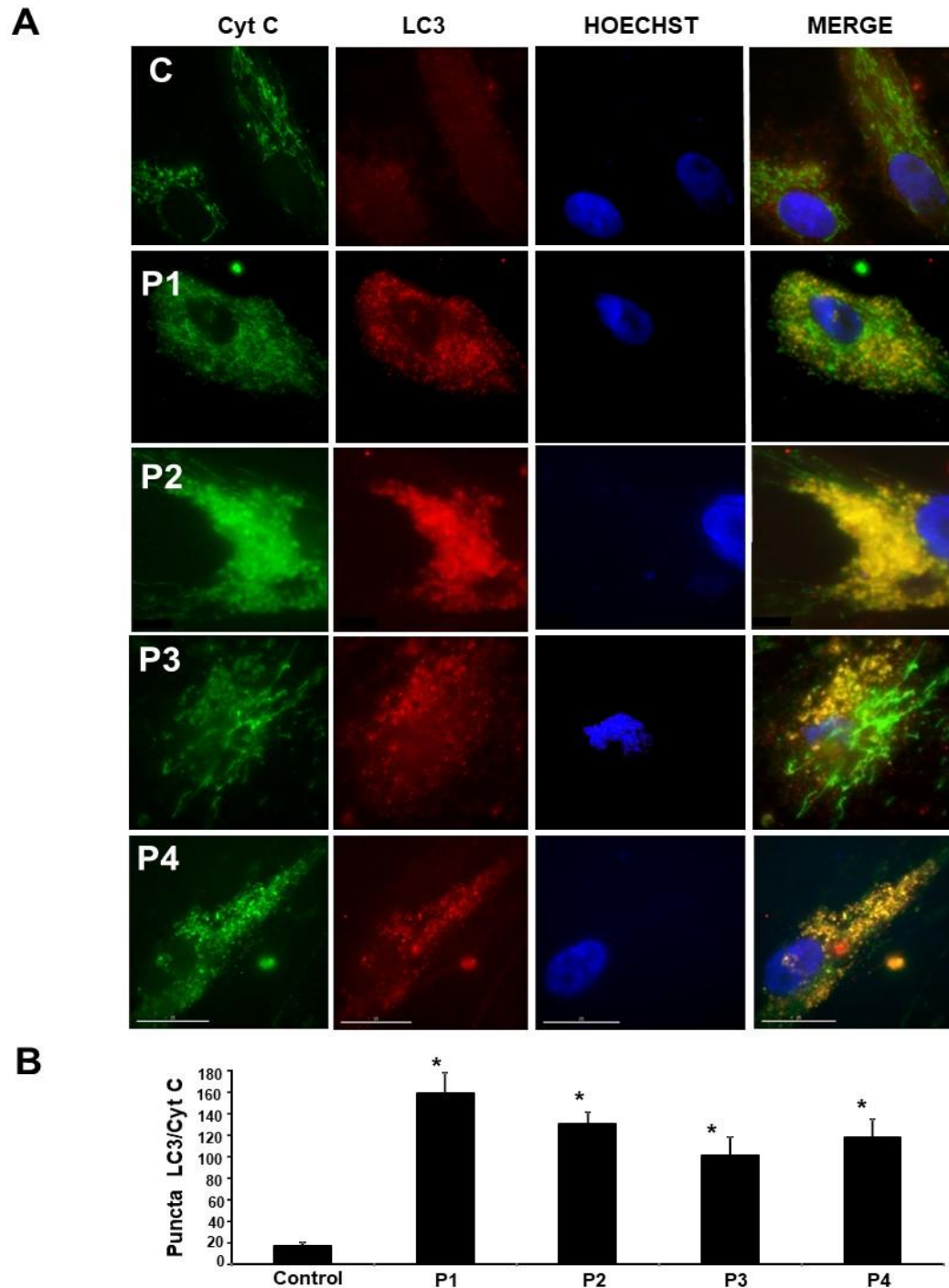


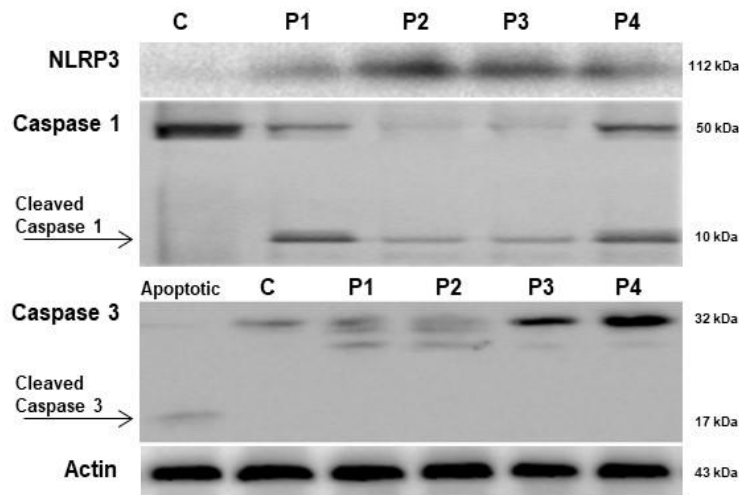
Figure 15. Autophagosome and mitochondria markers colocalization in FH fibroblasts. (A) Image analysis of LC3 and cytochrome c immunostaining in control and FH fibroblasts. Control and FH fibroblasts were fixed and immunostained with anti LC3 (autophagosome marker) and cytochrome c (mitochondrial marker) and examined by fluorescence microscopy. Nuclei were revealed by Hoechst staining. Scale bar=15 μ m. (B) Quantification of LC3/cytochrome c puncta in control and FH (n=100 cells). Data represent the mean \pm SD of 3 separate experiments. *p<0.01 between control and FH fibroblasts.

FH, Familial Hypercholesterolemia; LC3, Microtubule-associated proteins 1A/1B light chain 3B; SD, Standard Deviation

6.4. Inflammasome activation in FH fibroblasts

Recent data suggest that damaged mitochondria could induce inflammasome activation (Cordero et al., 2014; Gurung, Lukens, & Kanneganti, 2015). It has been described that NLRP3 (nacht Domain-, leucine-rich repeat-, and PYD-containing protein 3) is able to sense dysfunctional mitochondrion explaining the common association between mitochondrial damage and inflammatory diseases (Zhou, Yazdi, Menu, & Tschopp, 2011). Inflammation has also been described as a key process in the formation of the atherosclerotic plaque (Salminen, Ojala, Kaarniranta, & Kauppinen, 2012).

A



B

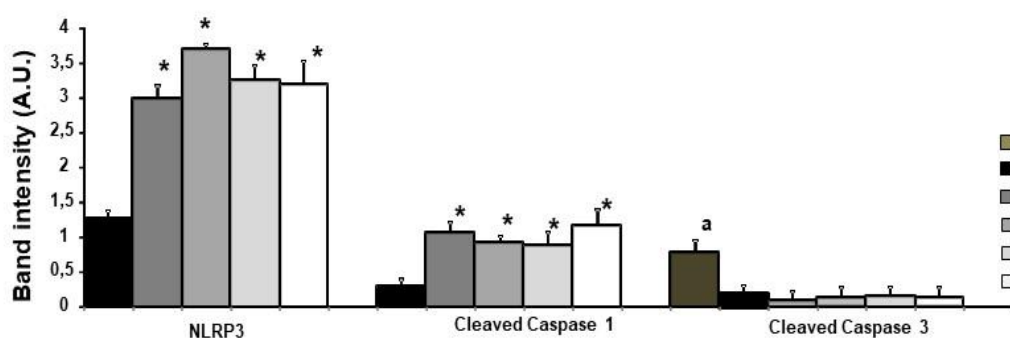
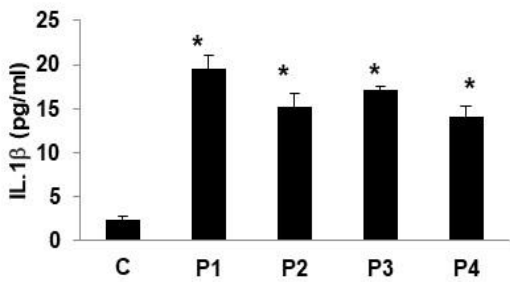
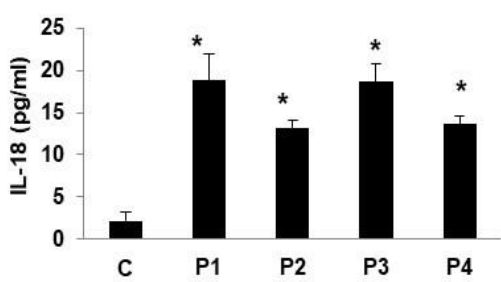


Figure 16. Inflammasome activation in FH fibroblasts. (A) Western blot analysis of NLRP3, caspase-1 and caspase 3 in control and FH fibroblasts. Actin was used as loading control. (B) Densitometric analysis of Western blottings. Data represent the mean±SD of three separate experiments. *p<0.01 between control and FH fibroblasts. ^ap<0.05 between apoptotic and control and FH cells. C: IL-1β and IL-18 levels in culture media of control and FH fibroblasts were determined by an ELISA assay. Data represent the mean±SD of three separate experiments. *p<0.01 between control and FH fibroblasts. A.U., arbitrary units.

C



D



FH, Familial Hypercholesterolemia; NLRP3, nacht Domain-, leucine-rich repeat-, and PYD-containing protein 3 ; SD, Standard Deviation; IL, Interleukin; ELISA, Enzyme-Linked Immuno Sorbent Assay; A.U., Arbitrary Unit

To assess inflammasome activation in patient fibroblasts, NLRP3 expression levels and Caspase-1 cleavage were analyzed by Western blotting as well as inflammatory cytokines levels, IL-1 β and IL-18, were measured by ELISA (Sharma & Kanneganti, 2016). Increased expression levels of NLRP3 and caspase-1-cleavage were found in patient fibroblasts (**Figure 16 A and B**) indicating inflammasome activation. Caspase-3 cleavage was also examined to rule out the possibility that inflammasome activation in FH fibroblasts was due to apoptosis activation. Furthermore, IL-1 β and IL18 levels in culture medium were significantly higher in FH fibroblasts (**Figure 16 C and D**).

6.5. Effect of CoQ₁₀ on FH fibroblasts

As FH fibroblasts showed a dysregulation of mevalonate pathway associated with increased cholesterol and reduced CoQ₁₀ biosynthesis, we next examined the effect of CoQ₁₀ treatment on cholesterol levels and the expression levels of several key proteins regulating this pathway. To that end, P1 and P3 fibroblasts were treated for 72 hours with 25 μ M CoQ₁₀ and intracellular cholesterol levels were quantified by both HPLC (**Figure 17 A**) and Filipin staining (**Figure 17 B**). Both determinations showed that cholesterol levels were significantly reduced in CoQ₁₀-treated FH fibroblasts, suggesting that CoQ₁₀ was able to correct mevalonate pathway. Furthermore, CoQ₁₀ treatment reduced significantly cytochrome c/LC3-II puncta (**Figure 17 C**) indicating improvement of mitochondrial function. The beneficial effects of CoQ₁₀ treatment on mutant cells bioenergetics was also confirmed using a mito-stress test assay using the XF24 extracellular flux analyzer (**Figure 18**).

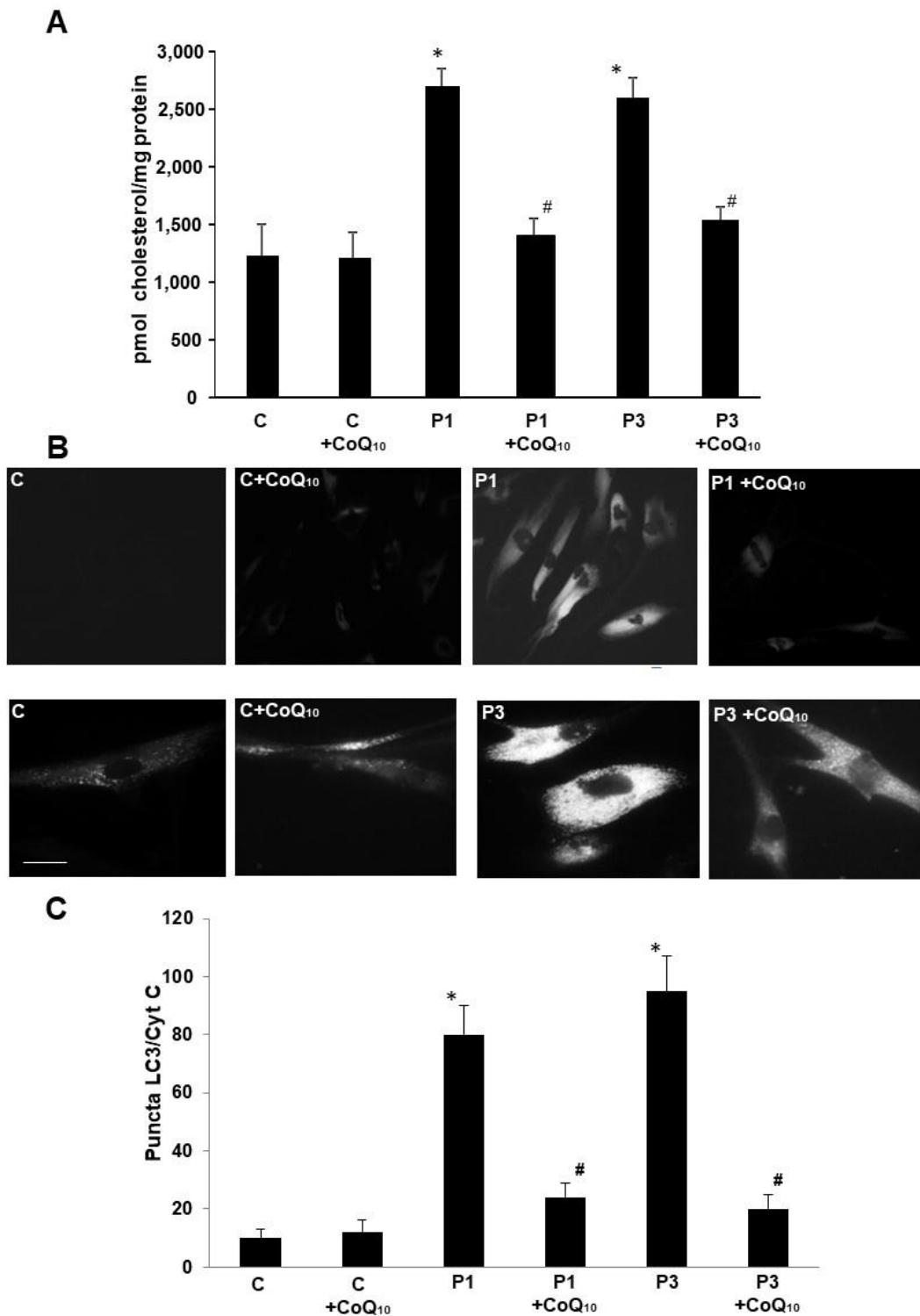


Figure 17. CoQ₁₀ treatment restores cholesterol levels and reduces mitophagy in FH fibroblasts. (A) Control and FH fibroblasts were treated with 25 μ M CoQ₁₀ for 72 hours. Cholesterol levels in control and FH fibroblasts were determined by hexane extraction and HPLC separation. (B) Filipin staining in control and FH fibroblasts treated with CoQ₁₀. (C) Quantification of puncta (LC3 and cytochrome c colocalization) in control and FH fibroblasts treated with CoQ₁₀. Control and FH fibroblasts were fixed and immunostained with anti-LC3 (autophagosome marker) and cytochrome c (mitochondrial marker) and examined by fluorescence microscopy. For control cells, the data are the mean \pm SD for experiments conducted on 2 different control cell lines. Data represent the mean \pm SD of 3 separate experiments. * p <0.01 between control and FH fibroblasts. # p <0.01 between the presence and the absence of CoQ₁₀ treatment.

CoQ₁₀, Coenzyme Q₁₀; FH, Familial Hypercholesterolemia; HPLC, High Pressure Liquid Chromatography; LC3, Microtubule-associated proteins 1A/1B light chain 3B; SD, Standard Deviation

6.6. CoQ₁₀ treatment restores altered pathways in FH fibroblasts

In order to explore the molecular mechanism underlying the beneficial effect of CoQ₁₀, the expression levels of several proteins regulating the mevalonate pathways were examined in control and FH fibroblasts. CoQ₁₀ treatment of patient fibroblasts induced AMPK (5' adenosine monophosphate-activated protein kinase) activation and increased PPAR- α (peroxisome proliferative activated receptor alpha) expression levels. This was associated with the restoration of COQ1, COQ2, COQ7 expression levels (**Figure 19** and **Figure 20**). Furthermore, CoQ₁₀ treatment decreased the expression levels of SREBP-2 (mature form), HMGCR and cholesterologenic enzymes such as squalene and lanosterol synthase. In contrast, CoQ₁₀ increased the expression levels of LDL-R and reduced the expression levels of PCSK9. Interestingly, CoQ₁₀ treatment also increased markedly LDL uptake by FH fibroblasts (**Figure 21A** and **21B**). The beneficial effects of CoQ₁₀ on LDL-R and COQ1 expression levels were dose dependent as is showed in **Figure 21C**. Moreover, the effect of CoQ₁₀ treatment on essential proteins for cholesterol sensing such as INSIG1, INSIG2 and SCAP was also examined. Surprisingly, the expression levels of INSIG1 and INSIG2 were markedly reduced in FH fibroblasts (**Figure 19** and **Figure 20**). Interestingly, the expression of both INSIGs was notably upregulated after CoQ₁₀ treatment. In contrast, SCAP expression levels were slightly increased in FH fibroblasts and they did not change significantly after CoQ₁₀ treatment (**Figure 19** and **Figure 20**).

To assess the role of AMPK activation in correcting mevalonate pathway under CoQ₁₀ treatment we examined the expression levels of essential regulatory proteins of this pathway in the presence or absence of compound C, a selective inhibitor of AMPK. The correction of the expression of mevalonate pathway enzymes by CoQ₁₀ was prevented by AMPK inhibition (**Figure 22** and **Figure 23**). Similarly, the effect of CoQ₁₀ on the expression of LDL-R, PCSK9 and NLRP3 was blocked by compound C (**Figure 22** and **Figure 23**). Taking together, these results suggest that the positive effects of CoQ₁₀ depend on AMPK activation.

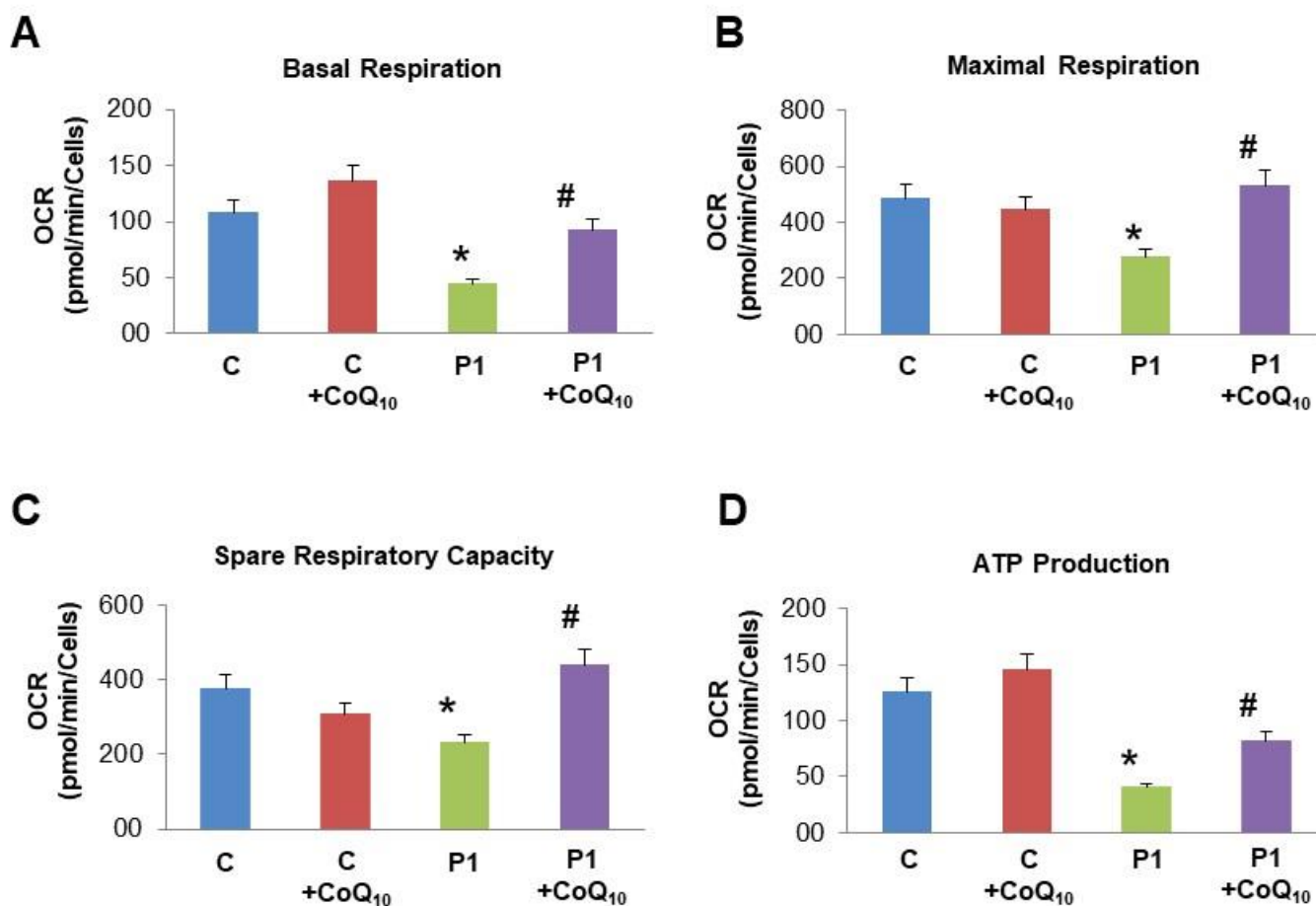


Figure 18. Effects of CoQ₁₀ treatment on cell bioenergetics. Basal (A) and maximal (B) respiration, Spare Respiratory capacity (C) and ATP production (D) were determined in control (C1) and FH fibroblasts (P1) by using the Seahorse analyzer. Control and FH fibroblasts were treated with 25 μ M CoQ₁₀ for 72 h. * p <0.01 between Control and FH fibroblasts. # p <0.01 between untreated and treated FH fibroblasts.

CoQ₁₀, Coenzyme Q₁₀; ATP, Adenosin triphosphate; FH, Familial Hypercholesterolemia

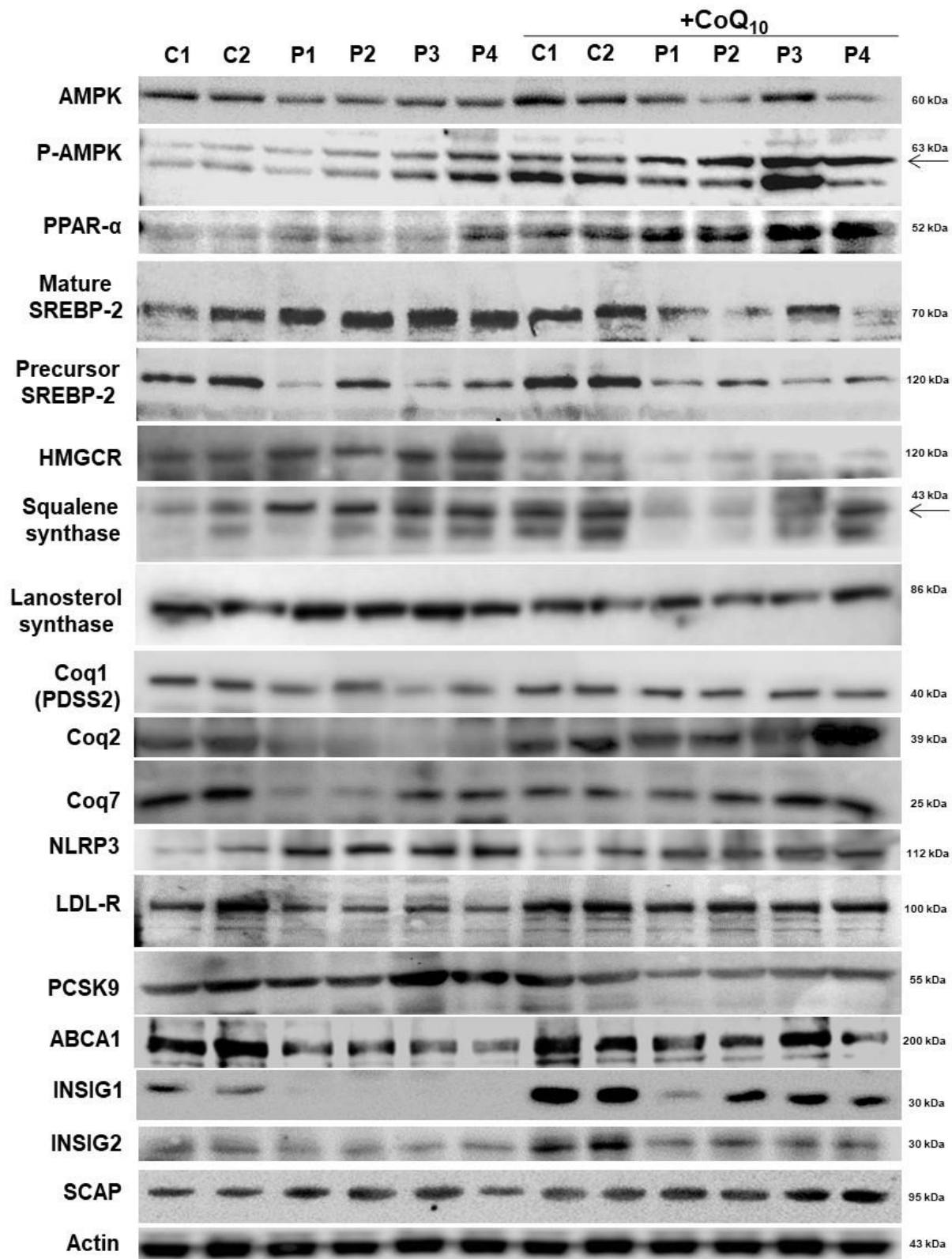


Figure 19. Effect of CoQ₁₀ treatment on several pathways regulating mevalonate pathway. Western blot analysis of SREBP-2 processing, AMPK activation, and expression levels of PPAR-α, HMGCR, lanosterol synthase, squalene synthase, COQ2, COQ7, NLRP3, LDL-R, PCSK9, ABCA1, INSIG1, INSIG2 and SCAP. Control and FH fibroblasts were treated with 25 μM CoQ₁₀ for 72 hours. A representative actin band is shown, although loading control was checked in every Western blotting.

CoQ₁₀, Coenzyme Q₁₀; SREBP-2, Sterol regulatory element-binding protein 2; AMPK, AMP-activated protein kinase; PPAR- α, Peroxisome proliferator-activated receptors; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; NLRP3, nacht Domain-, leucine-rich repeat-, and PYD-containing protein 3; PCSK9, Proprotein convertase subtilisin/kexin type 9; LDL-R, Low Density Lipoprotein Receptor; ABCA1, ATP-binding cassette transport; INSIG1, Insulin induced gene; SCAP, SREBP cleavage-activating protein; FH, Familial Hypercholesterolemia

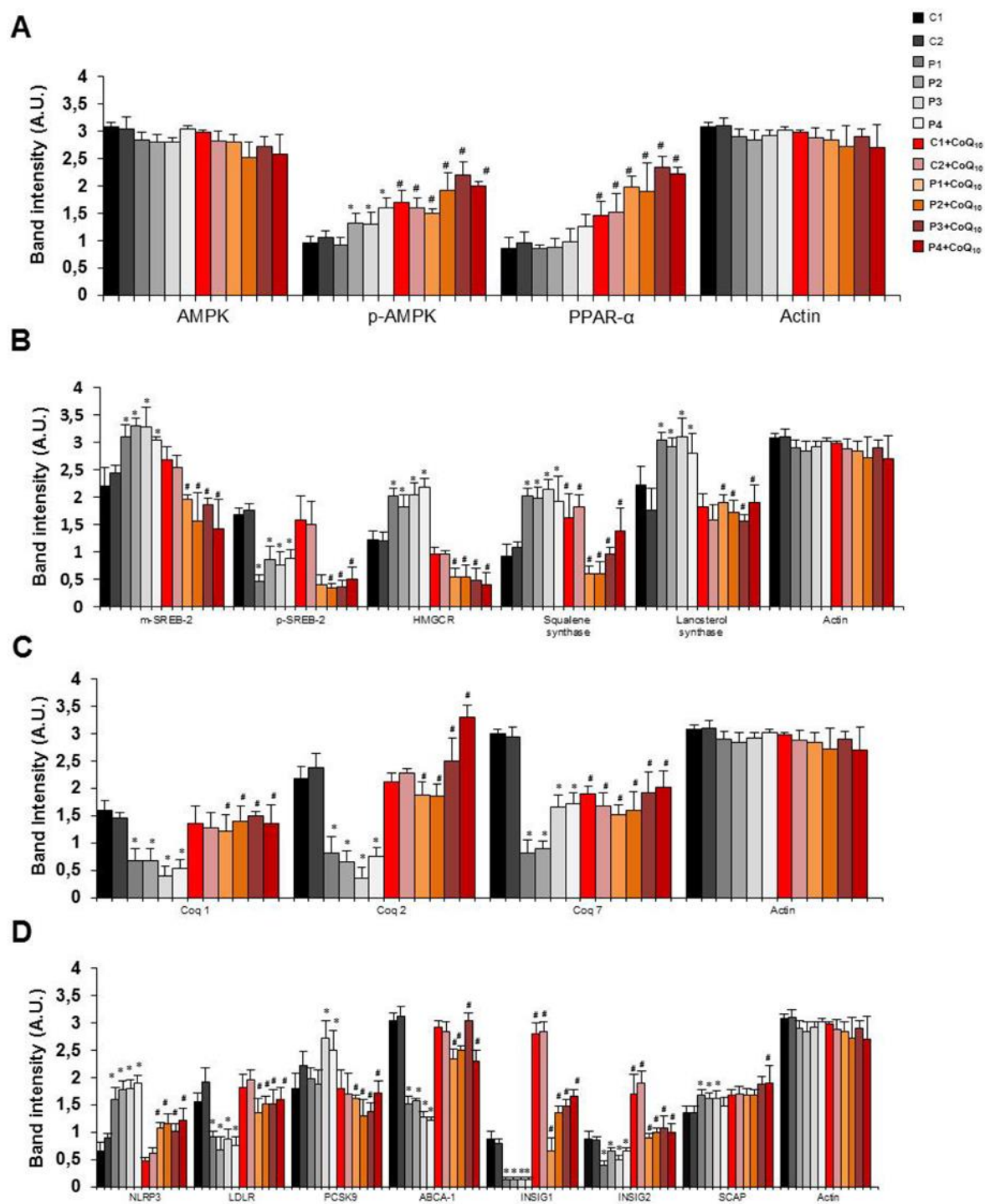


Figure 20. Densitometric analysis of Western blotting of Figure 19. (A, B, C and D) Data represent the mean±SD of three separate experiments. * $p < 0.05$ between control and FH fibroblasts. # $p < 0.05$ between the presence and the absence of CoQ₁₀ treatment.

SD, Standard Deviation; FH, Familial Hypercholesterolemia; A.U., arbitrary units; CoQ₁₀, Coenzyme Q₁₀

6.7. Cholesterol accumulation and mitochondrial dysfunction by silencing LDL-R in endothelial cells

To reproduce the pathological alterations observed in patient fibroblasts, LDL-R was silenced by siRNA in human endothelial cells. As expected, LDL-R silencing in endothelial cells induced a marked reduction of LDL uptake (

Figure 21 A and B) accompanied by up-regulation of cholesterogenic enzymes such as HMGCR, lanosterol synthase and squalene synthase, and down-regulation of biosynthetic CoQ₁₀ enzymes such as COQ1 (

Figure 21 C and D). LDL-R silencing also induced a marked down-regulation of INSIG1 and INSIG2 expression levels as well as a significant increase of SCAP expression levels. Accordingly, LDL-R silenced cells presented cholesterol accumulation (

Figure 21 E), mitochondrial depolarization (

Figure 21 F) and inflammasome activation assessed by increased NLRP3 levels (

Figure 21 A and B).

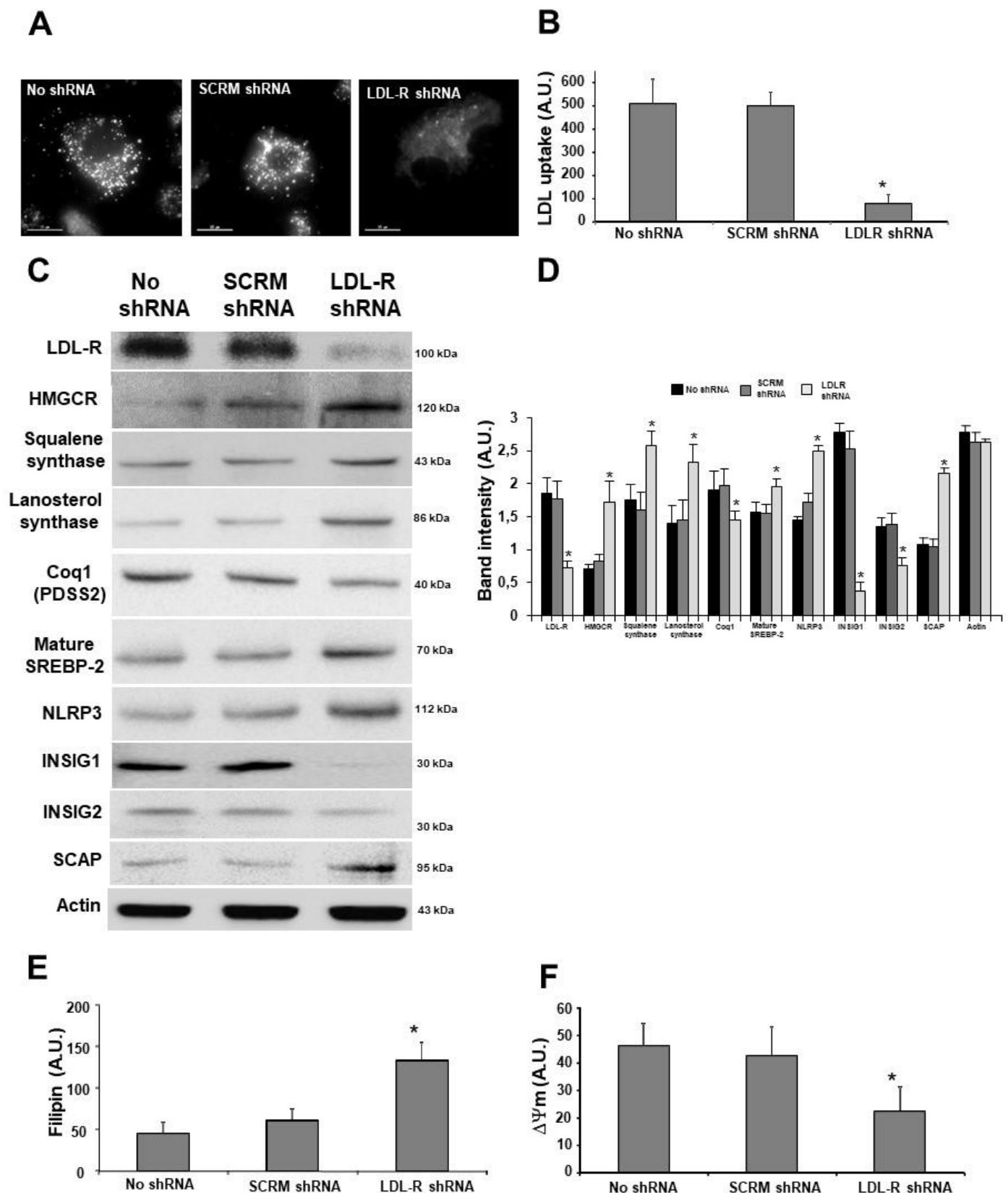


Figure 21. Effects of LDL-R silencing on human endothelial cells. (A) Control, control siRNA (SCRM), and LDL-R silenced endothelial cells were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy as described in Material and Methods. An impaired LDL-uptake was found in LDL-R silenced endothelial cells. (B) Quantification LDL-uptake by Image-J software. (C) Western blot analysis of LDL-R, HMGCR, squalene synthase, lanosterol synthase, COQ1, mature SREBP-2, NLRP3, INSIG1, INSIG2 and SCAP in cell extracts from control, control siRNA (SCRM) and LDL-R silenced endothelial cells. Actin was used as loading control. (D) Densitometry of Western blotting. (E) Quantification of cholesterol in control, control siRNA (SCRM), and LDL-R silenced endothelial cells by Filipin staining. (F) Mitochondrial dysfunction was addressed by measuring $\Delta\Psi_m$ by flow cytometry. * $p < 0.01$ between control and silenced cells.

LDL-R, Low Density Lipoprotein Receptor; SCRM, Control siRNA; LDL, Low Density Lipoprotein; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; SREBP-2, Sterol regulatory element-binding protein 2; NLRP3, nacht Domain-, leucine-rich repeat-, and PYD-containing protein 3; INSIG, Insulin Induced Gene; SCAP, SREBP cleavage-activating protein; $\Delta\Psi_m$, mitochondrial membrane potential; A.U., arbitrary units.

7. Discussion

In this work, we have examined the pathophysiology of FH in primary cultured fibroblasts derived from 4 patients harboring LDL-R mutations. As a consequence of defective LDL-R, FH fibroblasts showed a marked decrease in the incorporation of cholesterol mediated by LDL-R (**Figure 8** and **Figure 22**). Interestingly, intracellular cholesterol levels were considerably high in FH patients, suggesting that cholesterol biosynthesis was up-regulated. Since the seminal works of Brown and Goldstein is well known that defective binding of lipoproteins to cultured fibroblasts induces impaired regulation of HMGCR activity (Brown & Goldstein, 1974). This binding appears to be a required step in the process by which LDL normally suppresses the synthesis of HMGCR, the rate-controlling enzyme in cholesterol biosynthesis. The demonstration of a defect in LDL binding appears to explain the failure of lipoproteins to suppress the synthesis of this enzyme and hence may account for the overproduction of cholesterol that occurs in FH cells. Thus, increased intracellular lipogenesis in FH fibroblasts would be a consequence of a constitutive increase of enzymes involved in cholesterol synthesis due to defective extracellular cholesterol uptake.

In addition to elevated cholesterol levels and cholesterologenic enzymes expression, a significant decrease in CoQ₁₀ levels associated with down-regulation of proteins involved in CoQ₁₀ biosynthesis was also found in FH fibroblasts (**Figure 11** and **Figure 12**). These results suggest a possible imbalance in the mevalonate pathway that could affect the synthesis of both cholesterol and CoQ₁₀ which eventually provokes cholesterol overproduction/accumulation and secondary CoQ₁₀ deficiency. It is also plausible to think that a high demand for cholesterol would shift the intermediary substrates flow into one direction (steroidogenesis) and decrease a collateral pathway (CoQ₁₀ synthesis).

In turn, secondary CoQ₁₀ deficiency may impair mitochondrial function (Cotán et al., 2011). Thus, our data showed mitochondrial dysfunction associated with high ROS production, reduced ATP levels, mitochondrial depolarization and low activity of mitochondrial respiratory complexes in FH fibroblasts (**Figure 14** and **Figure 18**). These markers of mitochondrial dysfunction have been previously described in several disorders such as fibromyalgia, mitochondrial and lysosomal diseases as well as primary or secondary CoQ₁₀ deficiencies (Cordero et al., 2010; Cotán et al., 2011; De la Mata et al., 2012).

In addition, autophagy was examined in FH fibroblasts to determine if mutant cells manifested extensive mitophagy as it had been previously described in fibroblasts suffering from mitochondrial dysfunction (Cordero et al., 2010; Cotán et al., 2011; De la Mata et al.,

2012). Our results showed that autophagic genes and autophagic proteins were significantly increased in FH fibroblasts. Immunofluorescence examination also revealed colocalization of mitochondrial and autophagosome markers indicating mitophagy activation (**Figure 13**, **Figure 14** and **Figure 15**). Autophagic flux refers to the whole process of autophagy, including autophagosome formation, maturation, fusion with lysosomes, subsequent breakdown and the release of degraded molecules into the cytosol, representing the dynamic process of autophagy (Xiao-jie Zhang, Chen, Huang, & Le, 2013). Our results indicated that autophagy flux was also impaired in FH fibroblasts. This alteration could be responsible for the accumulation of engulfed mitochondria by autophagolysosomes in mutant cells.

Both lipid accumulation and mitochondrial dysfunction have been considered to play an important role for inflammation processes (Alcocer-Gómez et al., 2014; Gurung et al., 2015). Indeed, FH fibroblasts showed cholesterol accumulation, mitochondrial dysfunction and inflammasome activation (**Figure 16**). Inflammatory processes have been associated with the development of an atherosclerotic plaque, which is created by an oxidative modification of LDL when it is transported to the arterial wall (Madamanchi & Runge, 2007). According to our results, mitochondria dysfunction and CoQ₁₀ deficiency in FH cells can also play an important role in the pathophysiology of early atherosclerosis by contributing to increased production of free radicals and inflammation in the endothelium of blood vessels.

One of the most consistent hypothesis to explain the premature atherosclerosis (Chisolm & Steinberg, 2000) in FH patients postulates that the disease is due to oxidation of LDL (Witztum & Steinberg, 1991) by free radicals produced by circulating and vascular wall cells (Lamb, Wilkins, & Leake, 1992; Morel, DiCorleto, & Chisolm, n.d.). However, it is not clear where and how the conditions are set for the generation of oxidative stress in FH. Increased ROS production in isolated mitochondria from liver tissue, cardiac and brain and spleen mononuclear cells isolated from LDL-R knockout mice have been reported (Oliveira et al., 2005). Thus, the defect in LDL-R leads to two important proatherogenic effects: increased levels of oxidized LDL and imbalance of cellular redox state. This latter process would be responsible for local stress, which induces lipoprotein oxidation and mitochondrial damage (Vindis et al., 2005; Zmijewski et al., 2005). The resulting vicious cycle would cause cell death and progress of arteriosclerosis in hypercholesterolemia by the absence of LDL-R. This redox imbalance can be important in the pathogenesis of other diseases that also occur with increased hyperlipidemia and lipidogenesis such as diabetes, nephrotic syndrome, obesity, and metabolic syndrome (Vercesi, Castilho, Kowaltowski, & Oliveira, 2007). Within the vascular

wall, increased mitochondrial oxidative stress may contribute to the oxidation of lipoproteins, which, along with increased susceptibility to cell death may be a causal effect on the development of atherosclerotic lesions. In addition, oxidative stress in mitochondria and susceptibility to cell death can contribute to tissue injury of ischemia that occurs in vascular accidents (stroke) and heart attacks in FH.

In this project, we proposed that secondary CoQ₁₀ deficiency due to a constitutive dysregulation of mevalonate pathway may in part explain the generation of oxidative stress in FH cells. Furthermore, mitochondria dysfunction was associated with mitophagy and inflammasome activation, and many experimental studies have reported that NLRP3 inflammasome plays a crucial role in the progression of atherosclerosis (Karasawa & Takahashi, 2017). Thus, Duewell and colleagues showed that NLRP3-deficient bone marrow cells transplanted into atherosclerosis-prone low-density lipoprotein receptor-deficient mice had reduced atherosclerosis (Duewell et al., 2010).

Currently, statins are the main therapeutic option for lowering levels of total cholesterol and LDL-C. Statins act by inhibiting HMGCR and their development over the past 20 years has represented a breakthrough for the treatment of hypercholesterolemia (Jasińska, Owczarek, & Orszulak-Michalak, n.d.). Nowadays, the use of statins is well tolerated and they have a good safety profile (Bays, 2006). However many adverse effects such as hepatotoxicity, neuropathies, risk of increased incidence of cancer (Alsheikh-Ali, Maddukuri, Han, & Karas, 2007), and various forms of myotoxicity mainly from myalgia to rhabdomyolysis have been described. These deleterious manifestations of muscle occur in some studies in 1-7% of patients treated with statins and do not correlate with the effectiveness on the cholesterol lowering drug used (Baer, Miyamoto, & Denver, 2007; Christopher-Stine, 2006). The pathophysiological mechanisms of statin-induced myopathy are not fully known. One of the proposed mechanisms postulated that myopathy statins is due to a mitochondrial dysfunction due to inhibition of the mevalonate pathway which is essential for the synthesis of the isoprenoid chain of CoQ₁₀ (P. D. Thompson, Clarkson, & Karas, 2003). In the other hand, CoQ₁₀ is an essential cofactor in the mitochondrial electron transport (Crane, 2001), and mitochondria are essential to the normal functioning of high energy-demanding tissues such as muscles. Aftermarket studies have indicated that up 13.6% of patients treated with statins experience some degree of myopathy (Scott, Lintott, & Wilson, 1991). The diagnosis of the severity of secondary CoQ₁₀ deficiency and mitochondrial impairment in FH patients can be

important to prevent potential myotoxic effects caused by treatment with statins at high doses.

For all above reasons, alternative approaches in FH are based on the evidence that statins presumably affect also CoQ₁₀ biosynthesis that is already reduced by an imbalance in the CoQ₁₀/cholesterol biosynthetic pathway in FH. In addition, new therapies are necessary for patients who cannot reach the target LDL-C level when taking the maximum-tolerated dose of a statin or cannot tolerate them. Therefore, treatments focused on reducing intracellular cholesterol content and raising CoQ₁₀ levels would be appropriate for neutralizing mitochondrial damages and restoring the dysregulated cholesterologenic pathway in FH patients.

SREBPs are membrane-bound basic helix-loop-helix leucine zipper transcription factors that serve as master regulators of lipid homeostasis by regulating synthesis of cholesterol, fatty acids, and triglycerides (Jeon & Osborne, 2012). The three SREBP isoforms, SREBP-1a, SREBP-1c and SREBP-2, have different roles in lipid synthesis (Brown & Goldstein, 1998). *In vivo* studies using transgenic and knockout mice suggest that SREBP-1c is involved in fatty acid synthesis and insulin induced glucose metabolism (particularly in lipogenesis), whereas SREBP-2 is relatively specific to cholesterol synthesis. When sterol levels decrease by reduced uptake, as it is the case in FH cells, the precursor is cleaved to activate cholesterologenic genes and proteins and maintain cholesterol homeostasis (Horton et al., 2002). Release of membrane-bound SREBP requires SREBP cleavage-activating protein (SCAP) to escort SREBP from the endoplasmic reticulum to the Golgi for cleavage by site-1 and site-2 proteases. Thus, in FH fibroblasts the low cholesterol uptake provokes increased SREBP-2 maturation and consequently increased cholesterol biosynthesis. However is not clear why increased cholesterol biosynthesis persists despite FH cells contain high cholesterol levels. One plausible explanation is that FH cells are not able to sense cholesterol accumulation which depends on INSIG1 and INSIG2, proteins that interact with and retain SREBPs in the ER, inhibiting their activation (Dong et al., 2012) and negatively regulating HMGCR transcription (Peter J. Espenshade & Hughes, 2007; Sever et al., 2003a). Confirming this assumption, cholesterol and triglycerides are over-accumulated in INSIGs knockout mice liver (Engelking et al., 2005). Our results showed that INSIG1 was practically absent while INSIG2 was significantly reduced in FH fibroblasts indicating that cholesterol sensing in ER must be impaired. As a result, SREBP2 was constitutively activated and cholesterol was accumulated.

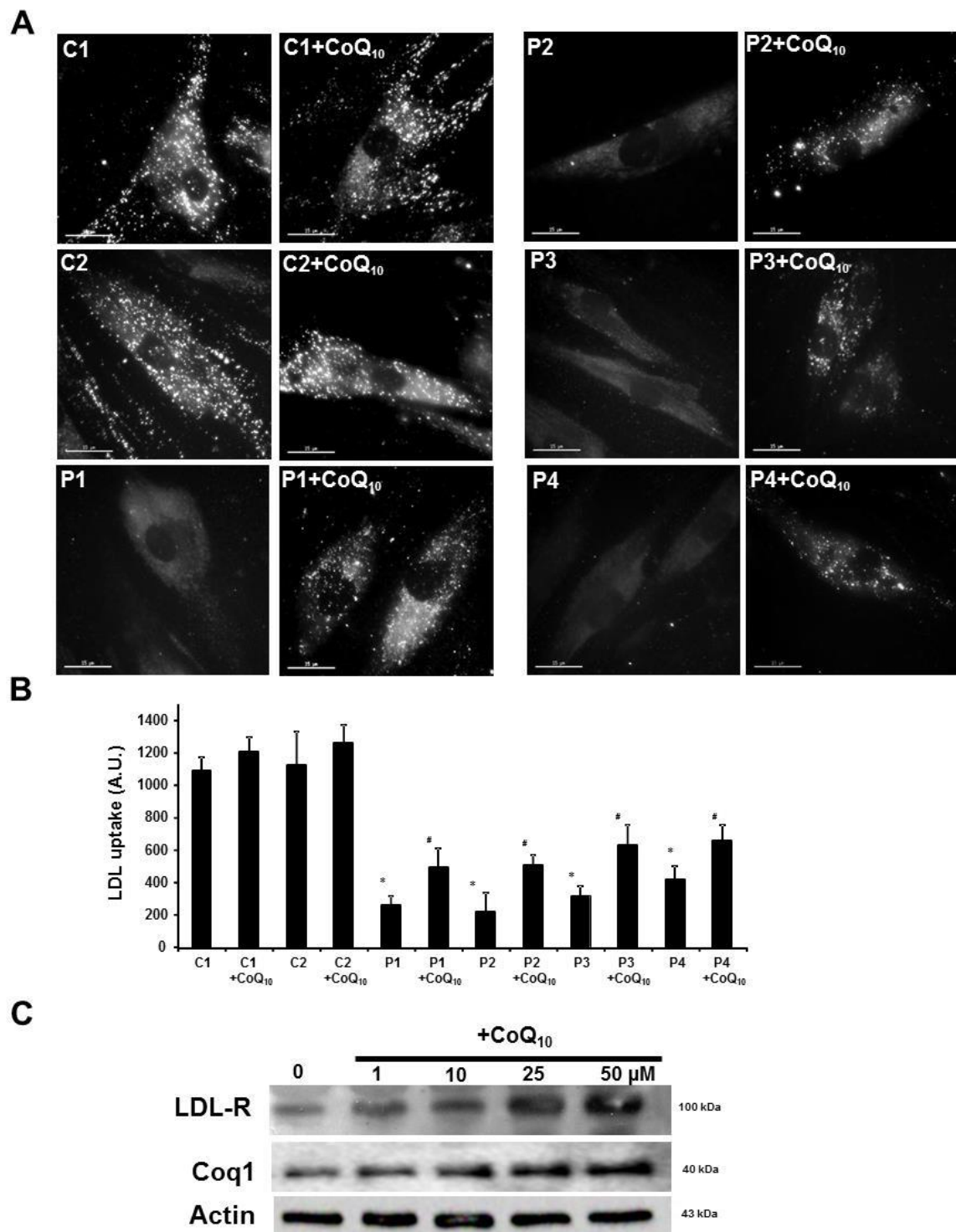


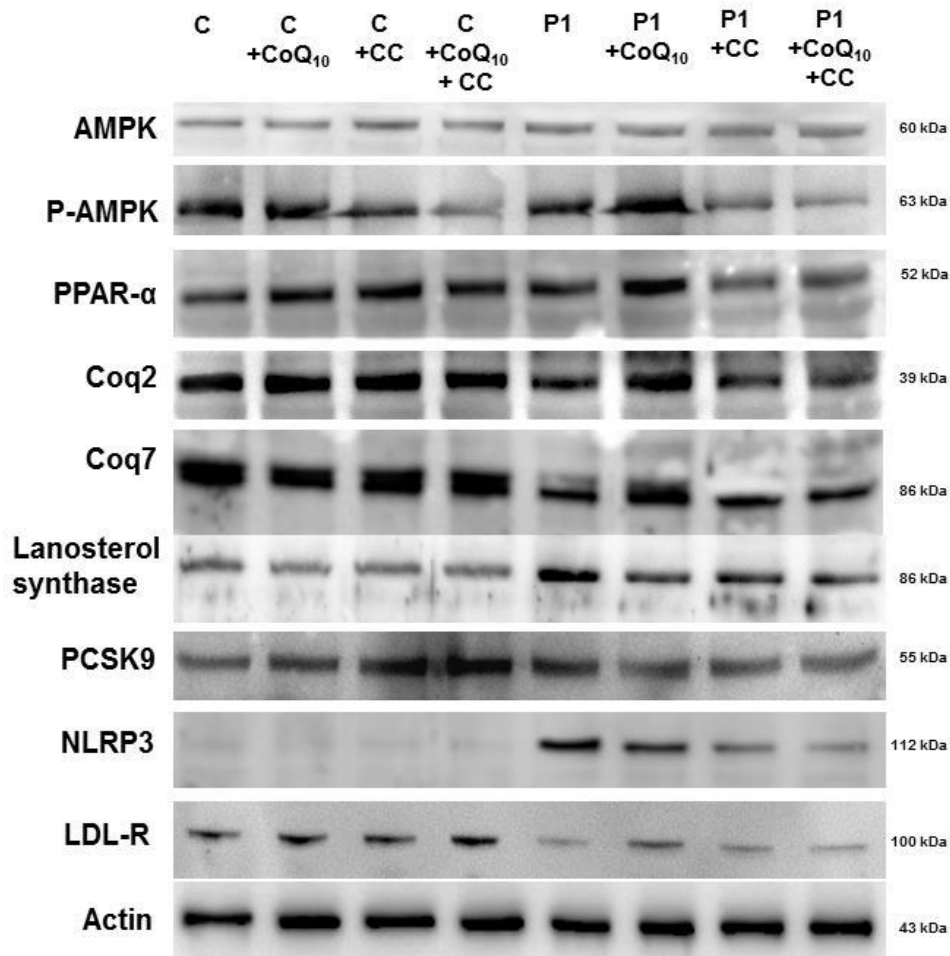
Figure 22. Effect of CoQ₁₀ treatment on FH fibroblasts. (A) Effect of CoQ₁₀ on LDL uptake. After 25 μ M CoQ₁₀ treatment for 72 hours, control and FH fibroblasts were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy. CoQ₁₀ treatment improves the impaired LDL-uptake in FH fibroblasts. (B) Quantification LDL-uptake by Image-J software. (C) Western blot analysis of LDL-R and Coq1 expression levels under increasing concentrations of CoQ₁₀ (1-50 μ M) in patient fibroblasts (P1). Data represent the mean \pm SD of three separate experiments. Actin was used as a loading control. * p <0.01 between control and FH fibroblasts. ^a p <0.05 between the presence and the absence of CoQ₁₀ treatment.

CoQ₁₀, Coenzyme Q₁₀; LDL, Low Density Lipoprotein; FH, Familial Hypercholesterolemia; SD, Standard Deviation; A.U., arbitrary units.

Studies have shown that the regulatory actions of INSIGs in cholesterol metabolism are critically dependent on the ratios of INSIG proteins to their targets SCAP and HMGCR (Sever et al., 2003a; Yang et al., 2002). Thus, over-expression of SCAP or HMGCR saturates endogenous INSIGs, and impairs the proper regulation of cholesterol synthesis (P. C. W. Lee, Liu, Li, & Debose-Boyd, 2007). Therefore, a high ratio of SCAP to INSIGs, as we found in FH fibroblasts, may diminish sterol sensitivity of SREBP processing. Conversely, when INSIGs levels rise under CoQ₁₀ treatment, SREBP processing can be inhibited by lower concentrations of sterols (Yang et al., 2002). These findings highlight the importance of SCAP-INSIGs ratios in normal sterol-regulated processing of SREBPs in FH cells.

Our results also showed that both increased cholesterol levels and mitochondrial dysfunction can be restored by treatment with CoQ₁₀, a well-known player in cellular bioenergetics (**Figure 17, Figure 18, Figure 19, Figure 22 and Figure 23**). CoQ₁₀ has been reported to induce AMPK activation (Garrido-Maraver et al., 2015) and PPAR- α expression via the calcium-mediated AMPK signal pathway (S. K. Lee et al., 2012). CoQ₁₀ increases the expression of PPAR- α at both the mRNA and protein levels. Furthermore, knock down of AMPK with siRNA or inhibition of AMPK using the AMPK inhibitor compound C blocked CoQ₁₀-induced expression of PPAR- α , indicating that AMPK plays a critical role in PPAR- α induction. On the other hand, AMPK has been reported to inhibit the cleavage and transcriptional activation of SREBP-2, via direct phosphorylation (Li et al., 2011). Additionally, PPAR- α can inhibit cholesterol biosynthesis via reduction in SREBP-2 maturation (König et al., 2007). This, in turn, may reduce the expression levels of HMGCR, squalene synthase and other cholesterologenic proteins. Our results suggest that CoQ₁₀ could reduce cholesterol biosynthesis through AMPK-mediated PPAR- α stimulation. Interestingly, CoQ₁₀ treatment increases the expression levels of COQ1, COQ2 and COQ7, which have been reported to be under PPAR- α regulation (Bentinger et al., 2008). In addition, CoQ₁₀ treatment increased INSIG1 and INSIG2 expression levels which are also regulated by PPAR- α (König et al., 2007; J.-H. Lee et al., 2017). This restoration of INSIGs expression may allow a correct detection of cholesterol levels in ER membranes and proper feedback inhibition of the cholesterologenic pathway. All the beneficial effects of CoQ₁₀ on mevalonate pathway were accompanied by improvement of cell bioenergetics and reduction of mitophagy.

A



B

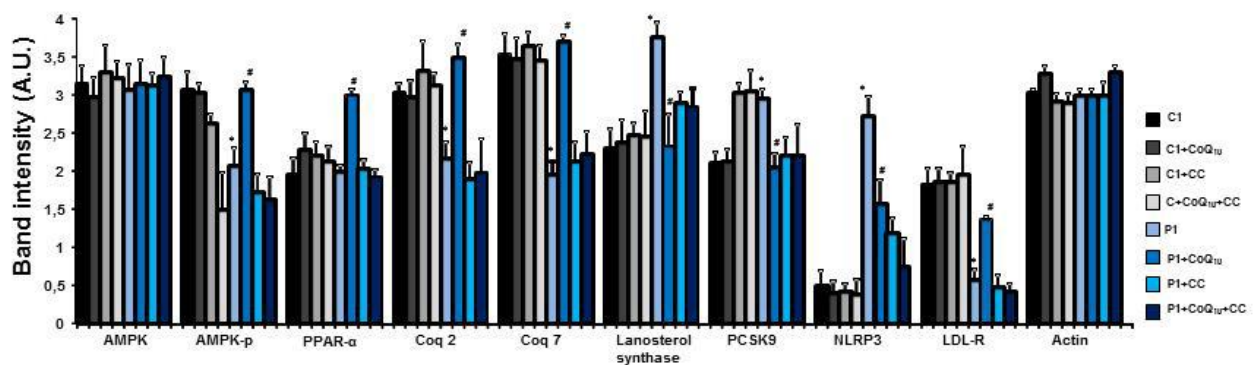


Figure 23. Effect of AMPK inhibition by compound C on CoQ₁₀ treatment. (A) Control and FH fibroblasts were treated with 25 μ M CoQ₁₀ in the presence of 10 μ M compound C (CC), an AMPK inhibitor, for 72 hours. Western blot analysis of AMPK, PPAR- α , Coq2, Coq7, lanosterol synthase, PCSK9 and NLRP3. Actin was used as a loading control. (B) Densitometry of Western blotting. * p <0.05 between control and FH fibroblasts. # p <0.01 between the presence and the absence of CoQ₁₀ treatment.

AMPK, AMP-activated protein kinase; CC, compound C; CoQ₁₀, Coenzyme Q₁₀; PPAR- α , peroxisome proliferator-activated receptors alpha; PCSK9, Proprotein convertase subtilisin/kexin type 9; NLRP3, night Domain-, leucine-rich repeat-, and PYD-containing protein 3; A.U., arbitrary units

Several studies have reported that AMPK activation reduced lipid synthesis by restraining SREBP activity and led to fatty acid oxidation in the liver to control hepatic energy metabolism (Viollet et al., 2006). On the other hand, CoQ₁₀-mediated activation of AMPK and/or PPAR- α may induce LDL-R upregulation as previously reported (Brusq et al., 2006; Huang et al., 2009). Although, it is known that LDL-R gene promoter activity is enhanced through the proteolytic activation of SREBP-2 and SREBP-1 (Yashiro, Nanmoku, Shimizu, Inoue, & Sato, 2012), our results showed that LDL-R expression and LDL uptake were markedly increased after CoQ₁₀ treatment despite reduced maturation of SREBP-2 (**Figure 22**). These findings suggest that LDL-R expression may be regulated by other molecular mechanisms possibly mediated by AMPK and/or PPAR- α . In agreement with these results, Schmelze et al. found that treatment with the reduced form of CoQ₁₀ in humans induced characteristic gene expression patterns, which were translated into reduced LDL-C levels (Schmelzer et al., 2011). Other report also suggested that CoQ₁₀ improved the hypolipemiant action of statins (Kang, Yang, Kang, Ryou, & Kang, 2012). CoQ₁₀ has been intensively implicated in protecting against chronic diseases, especially atherosclerosis (Garrido-Maraver et al., 2014). As increased ROS levels can modify cell-signaling proteins (including transcription factors and ion channel proteins) and have pathological consequences particularly in endothelial cells, it has been proposed that the antioxidant properties of CoQ₁₀ may also contribute to its beneficial effects on atherosclerosis (B.-J. Lee, Huang, Chen, & Lin, 2012a, 2012b).

In addition, CoQ₁₀ has been proposed to exert its atheroprotective effects partially by promoting miRNAs-mediated cholesterol efflux by inducing ABCA1 and ABCG1 expression (D. Wang et al., 2014; Yan et al., 2015) which have been shown to be critically involved in cholesterol and phospholipid efflux in macrophages, hepatocytes, and intestinal mucosa cells (Kerr, Haider, & Gelissen, 2011; Velamakanni, Wei, Janvilisri, & van Veen, 2007). Supporting these results, we found that CoQ₁₀ treatment of FH fibroblasts markedly induced ABCA1 expression.

The therapeutic effect of CoQ₁₀ in cardiovascular diseases has been reported to be caused by improving cardiovascular function, preventing LDL oxidation (Belardinelli, Tiano, & Littarru, 2008), and inhibiting atherosclerotic processes (Singh et al., 2003). There are also evidences showing that CoQ₁₀ has a positive effect on atherosclerotic processes improving endothelial function indices (Adarsh, Kaur, & Mohan, 2008; Tiano et al., 2007). In a study performed by Huynh and co-workers in diabetic patients, CoQ₁₀ decreased oxidative stress, improved lipid profiles, and remodeled left ventricle function (Huynh et al., 2012). Moreover, it

has been shown that oral prescription of CoQ₁₀ can decrease atherosclerotic damages in ApoE-deficient mice (Witting et al., 1999).

Our results illustrate that dysregulated mevalonate pathway associated with high intracellular cholesterol content and mitochondrial dysfunction as well as extensive mitophagy and inflammasome activation may participate in FH pathophysiology (**Figure 24**).

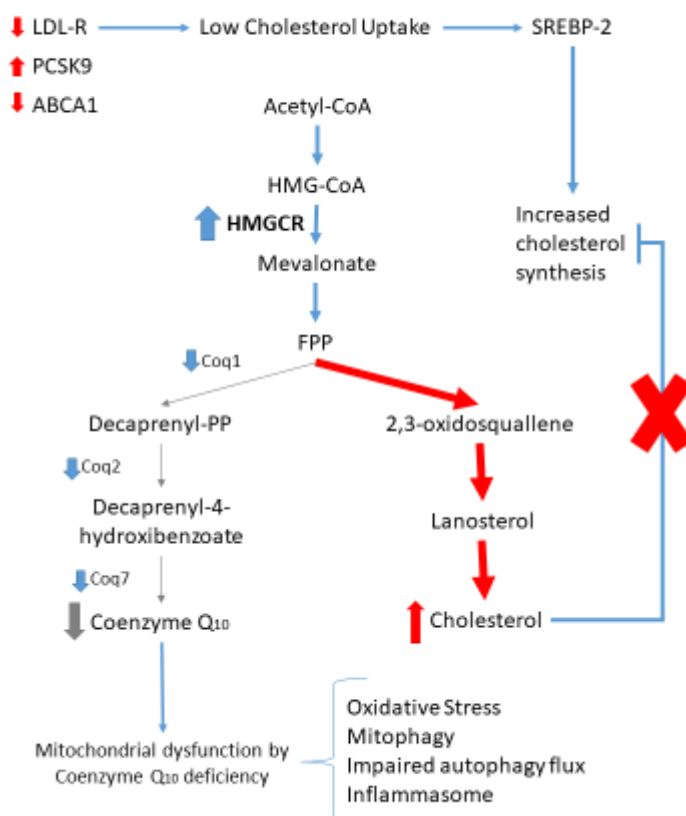


Figure 24. Working model of FH pathophysiology. Low expression of LDL-R results in dysregulated mevalonate pathway which leads to cholesterol accumulation and secondary CoQ₁₀ deficiency. As a consequence, mitochondrial dysfunction produces oxidative stress and mitophagy and inflammasome activation.

FH, Familial Hypercholesterolemia; LDL-R, Low Density Lipoprotein Receptor; CoQ₁₀, Coenzyme Q₁₀

AMPK activation by CoQ₁₀ treatment reduced SREBP-2 activation and cholesterol content in FH fibroblasts (**Figure 25**). This finding could be of great interest because AMPK activation has been proposed as a valuable approach to target lipid disorders (Ruderman & Prentki, 2004).

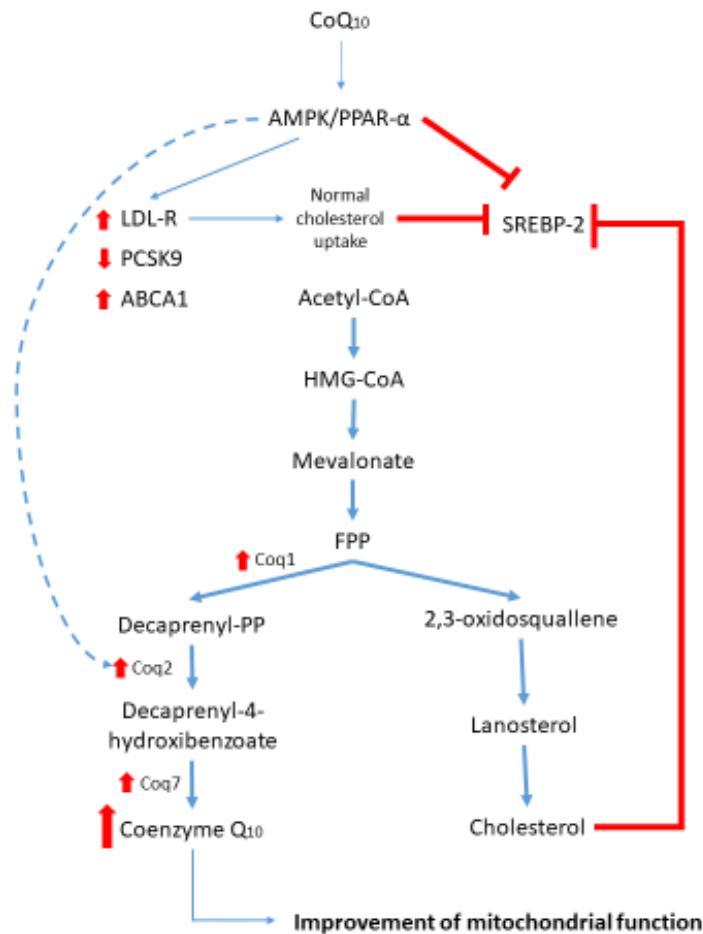


Figure 25. Working model of the impact of CoQ₁₀ treatment in FH. CoQ₁₀ treatment can correct both altered mevalonate pathway and mitochondrial function in FH fibroblasts. CoQ₁₀ causes a significant increase expression of LDL-R and ABCA1 accompanied by downregulation of PCSK9. In addition, CoQ₁₀ restores INSIG1 and INSIG2 expression levels and allows the feedback inhibition of SREBP-2 activation when cholesterol levels are high. The beneficial effects of CoQ₁₀ on mitochondrial function can also be attributed to CoQ₁₀ biosynthetic pathway up-regulation.

CoQ₁₀, Coenzyme Q₁₀; FH, Familial Hypercholesterolemia; LDL-R, Low Density Lipoprotein Receptor; ABCA1, ATP-binding cassette transporter; PCSK9, Proprotein convertase subtilisin/kexin type 9; INSIG, Insulin Induced Gene; SREBP-2, Sterol regulatory element-binding proteins

Furthermore, CoQ₁₀ treatment improved mitochondrial function, up-regulated LDL-R expression levels and restored INSIGs expression in FH fibroblasts. Its underlying mechanism is proposed to be mediated by the activation of AMPK and the concomitant up-regulated expression of PPAR- α . Therefore, CoQ₁₀ can be considered as a promising adjuvant pharmacotherapy of FH with the putative benefit of improving mitochondrial function and preventing inflammation. However, these results need to be confirmed using a larger sample size as well as in other models of FH.

8. Conclusions

1. Fibroblasts derived from patients with familial hypercholesterolemia caused by mutations in LDL-R show low expression LDL-R expression levels, defective LDL-C uptake and increased intracellular cholesterol biosynthesis.
2. Intracellular cholesterol accumulation in mutant fibroblasts is associated with constitutive SREBP-2 activation and low expression levels of INSIG1 and INSIG2, two essential proteins involved in cholesterol sensing.
3. LDL-R mutant fibroblasts show a dysregulated mevalonate pathway accompanied by increased cholesterologenic genes expression and decreased CoQ₁₀ biosynthesis genes expression.
4. Defective CoQ₁₀ biosynthesis in mutant fibroblasts induce mitochondrial dysfunction associated with increased oxidative stress, and reduced ATP production and mitochondrial mass.
5. Altered mitochondrial function in LDL-R mutant fibroblasts was associated with mitophagy and inflammasome activation accompanied by increased IL-1 β and IL-18 production.
6. LDL-R gene silencing in endothelial cells (EA.hy926) reproduce the cellular alteration of patient-derived fibroblasts. Silenced endothelial cells show reduced LDL-C uptake, altered cholesterologenic and CoQ₁₀ gene expression and intracellular cholesterol accumulation.
7. Supplementation of mutant fibroblasts with CoQ₁₀ correct most of the pathophysiological alterations in mutant fibroblasts such as LDL-R expression levels, dysregulated mevalonate pathway, intracellular cholesterol accumulation, mitochondrial dysfunction and increased mitophagy and inflammasome activity.
8. Fibroblast cell cultures derived from patients with familial hypercholesterolemia are useful cellular models for understanding pathophysiological alterations and the evaluation of therapeutic strategies.

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